EFFECTS OF HYPERGLYCEMIA AND CAFFEINE ON EARLY EMBRYOGENESIS IN WHOLE RAT EMBRYO CULTURE

BY

CHIU PUI YU

A thesis submitted to
Division of Obstetrics and Gynaecology
Faculty of Medicine
for the degree of
Master of Philosophy

July 2001

UL


香港中文大學
圖書館系統
02 MAY 2002
UNIVERSITY
LIBRARY SYSTEM
THE CHINESE UNIVERSITY OF HONG KONG

Abstract

Hyperglycemia is a well-known teratogen. It is one of the most common maternal illnesses resulting in congenital malformations. The incidence of maldevelopment among infants of hyperglycemic mothers is 3-4 times higher than in the normal population. Apart from congenital malformations, hyperglycemia accounts for approximately 4.73% of all perinatal deaths. The mechanism through which excess glucose results in tissue damage is still not precisely defined. The standpoint of this thesis is that oxidative stress may be the vital mechanism responsible for hyperglycemia-induced teratogenesis. The evidence that factors have been found associated with hyperglycemia, which can increase the production of free radicals supports this hypothesis.

Caffeine is one of the most popular dietary sources. Series of experiments and trials in past have confirmed that caffeine is a week teratogen in rodents. Its teratogenic effect in humans is still an open question. Apart from acting as a teratogen, caffeine has also been demonstrated to have antioxidant activity, which is as effective as glutathione and significantly more effective than ascorbic acid. Caffeine exerts its antioxidative ability in a dose-dependent fashion.

In the present study, we investigated the effect of addition of caffeine on hyperglycemia-induced teratogenesis with the use of a whole rat embryo culture model. We assessed embryos after culture with morphological scoring, 8-isoprostaglandins F2α measurement and protein content assay.

Our results reaffirmed the dose dependent teratogenicity of hyperglycemia on embryonic development in the phase of organogenesis, and successfully demonstrated that hyperglycemia induced over-production of free radicals significantly and dose-dependently. In the

caffeine study, the threshold teratogenic level of caffeine was found to be 30-60μg/ml. In our combined effect study, addition of teratogenic dose of glucose and sub-teratogenic dose of caffeine, we observed a significant improvement in both morphology and pro-oxidant activity in embryos. We propose that caffeine acts as effective antioxidant at sub-teratogenic doses to scavenge free radicals induced by hyperglycemia, and hence diminishes the degree of malformation caused by hyperglycemia.

摘要

高血糖症是一種已知的致畸原。它亦是其中一種最普遍引致先天性畸形的妊娠期疾病。由高血糖母親所產下的嬰兒中，他們患有畸形發展的機會比正常情況下高三至四倍。除此之外，高血糖症亦導致百分之四點三七的圍產期死亡。直至現在，仍未有一個明確的機制解釋高血糖症組織破損的原理。本論文認爲導致應激反應是一個主導高血糖致畸的機制。以上推論是由已知高血糖引致過量氧游離基所支持。

咖啡因是其中一種全球性的飲食源。過往一連串的實驗証明咖啡因在嚙齒類動物作爲一種弱致畸原。對人類而言，咖啡因是否引致胚胎發育異常仍具爭議。咖啡因除可致畸外，還同時擁有抗氧化活力。其抗氧化作用可媲美穀胱甘 ，甚至超越抗壞血酸。咖啡因抗氧化作用呈劑量相關性。

本研究中，我們希望利用全鼠胚體外培殖法去瞭解咖啡因對血糖高致畸形的影響。培殖後的胚胎進行以下評估：（一）發生學形態指數；（二）異列腺素 – F2 甲型的量度；（三）蛋白量的檢定。

實驗結果再次肯定劑量相關性高糖水平致畸作用，此作用主要影響胚胎的器官形成發育期。此外，結果亦成功地證明高糖濃度誘導過量游離基的產生，並呈顯注性及劑量相關性。咖啡因研究證實其致畸閾劑量爲每毫升三十微克至六十微克。在混合作用研究中，用低於咖啡因致畸閾劑量及高糖致畸劑量，我們觀察到胚胎在形態指數及氧激化水平均有明顯而良好的改善。是項研究證明少量的咖啡因透過抗氧化機制去消除由高血糖引致的過量游離基，從而達至胚胎育的好轉。

Acknowledgement

I would like to take this opportunity to send my sincere thanks to my supervisor, professor Lau Tze Kin. First of all, I would like to express my cordial gratitude to his insightful advice to this project- from the beginning of research design, technical system set up, to the data analysis and thesis writing. His invaluable comments and expert views build up my sense of logical thinking. Besides, I feel very grateful to his patient training and guidance within these two years, which benefit me in the coming future. I also want to acknowledge his provision of funds for the research project. Last but not least, his persistent and sincere care would be always impressive in my hearts. I know for certain that if it is not he, my project and this thesis would never be.

I also feel grateful to my parents for their patience, support and sacrificial love in my years of study. Further, special compliments are sent here to my friends who give me encouragement and support for this thesis. Thanks are also expressed to my colleagues and supporting staff in the Department of Obstetrics and Gynaecology CUHK, those in Animal House CUHK as well as those in Lee Hysan Clinical Research Laboratories.

I thank God for giving me the chance to study in the last two years. He gives me health, bravery, wisdom and strength to accomplish this task. In the coming days, I will continue to follow and put my trust in him. For in his time, he makes all things beautiful.

Table of Contents

Section III: Results

Section IV: Discussion and Conclusions

List of Tables

List of Figures

List of Abbreviations

cAMP	cyclic Adenosine Monophosphate
DM	Diabetes Mellitus
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
EIA	Enzyme-linked Immunoassay
ESR	Electron Spin Resonance
GCMS	Gas Chromatography Mass Spectrometry
GDM	Gestational Diabetes
IDD	Insulin-dependent Diabetes
IGT	Impaired Glucose Tolerance
NADH	Nicotinamide Adenine Dinucleotide
NIDD	Non-insulin-dependent Diabetes
PG	Prostaglandin
RDS	Respiratory Distress and Syndrome
ROS	Reactive Oxygen Species
SD	Spargue Dawley

Section I: Introduction

Chapter 1: Overview

Diabetes mellitus (DM) is a major health problem worldwide, affecting individuals from almost all populations and age groups. Epidemiological studies have shown that about 60 million people suffer from diabetes in the world (Global health situation 1993) and this number is constantly rising (King & Rewers 1991). The basic pathological feature of diabetes mellitus is hyperglycemia, which results in complications involving virtually every organ and system of the affected individuals (Sussman et al 1982; Omachi 1986; Harris et al 1993). The adverse effects of diabetes mellitus during hyperglycemia on reproduction are well known (Kitzmiller et al 1996; Lowy 1993; Hanson et al 1990), including a significant increase in incidence of congenital malformations amongst the offsprings (Malins 1979; Molsted-Pedersen & Pedersen 1985). Previous studies supported that this hyperglycemia-induced teratogenicity is, at least partly, mediated through the overproduction of free-oxygen radicals (Eriksson & Borg 1991; Eriksson & Siman 1996; Sivan et al 1996; Siman & Eriksson 1997; Wentzel & Eriksson 1998; Forsberg et al 1998; Trocino et al 1995; Yang et al 1997).

Caffeine is one of the most popular psychoactive compounds used worldwide (ICO 1991; Gilbert 1984). It is present in many dietary sources, in particular coffee (Bunker & McWilliams 1979; Burg 1975). Caffeine in high concentration may be teratogenic (Elmazar et al 1982; Ikeda et al 1982; Smith et al 1987; Collins 1979). On the other hand, caffeine has also been shown to possess antioxidant property (Devasagaam & Kesavan 1996; Shi & Dalal 1991). It is therefore interesting and clinically important to investigate the interaction between hyperglycemia and caffeine on

embryogenesis, which is the main objective of this thesis.

In the following two chapters, previous studies and evidence for hyperglycemia-induced teratogenesis, and on caffeine as a teratogen as well as an antioxidant will be reviewed. The research question will be discussed in chapter 4. The study design, methodology and results will be detailed in chapter 5, 6 and 7 respectively. Further discussions will be detailed in chapter 8, and the last chapter will conclude the findings, significance of the thesis, and its implication for future studies.

Chapter 2: Teratogenic Effects of Hyperglycemia

2.1 What is Hyperglycemia?

In Greece, 'hyper-' means 'above' whereas 'glycemic' relates to glucose levels. Hyperglycemia refers to a state in which the blood glucose level is higher than normal (Lawrence et al 1989).

Blood glucose level in pregnancy is tightly controlled by the interactions of hormones including oestrogen, progesterone, free cortisol, prolactin, insulin and glucagon. Many organs, including the ovary, fetal adrenal cortex, placenta, anterior pituitary, maternal adrenal cortex, and pancreas are involved in the production and control of these hormones.

Hyperglycemia may occur when the body has insufficient insulin (Type I or insulin-dependent) or when the body cannot use insulin properly (Type II or non-insulin-dependent). Pregnant women need more insulin to maintain normal carbohydrate metabolism because most fetal-placenta hormones have anti-insulin effects. If the woman is unable to maintain higher insulin production to meet the demand she may become diabetic.

In 1968, Emanuel reported that the normal fasting levels of glucose in each milliliter of human adult blood should be 0.87± 0.06 mg, while in 1980, the World Health Organization (WHO) suggested that a fasting blood glucose levels of more than 1.44g/ml should be regarded as DM. Freinkel et al in 1986 reported that the equivalent level in rats was approximate to 1.2 mg/ml.

2.2 Teratogenic Effects of Hyperglycemia

In Greece, 'Terat-' refers to monsters. Teratology is the branch of embryology and pathology that deals with abnormal developments and congenital malformations. Similarly, teratogenesis means induction of congenital deformity (Lawrence et al 1989).

2.2.1 Human Studies

Hyperglycemia or diabetes mellitus is known to adversely affect human reproduction. It is associated with a reduction in fertility potential and a significant increase in the incidence of abortion (Miodovnik et al1985), preterm labor (Jacobson & Cousins 1989; Rosenn et al 1993; Mimouni et al 1988), intra-uterine death (Drury et al 1977; White 1971), stillbirth (Jovanovic & Peterson 1980), and macrosomia (Oates et al 1980). Neonates born to mothers with diabetes mellitus are at higher risk of respiratory distress syndrome (RDS) (Nogee et al 1993), hypoglycemia (Druzin et al 1980), hyperbilirubinemia (Taylor et al 1963) and birth trauma (William 1995). Last, but not the least, hyperglycemia or diabetes mellitus is associated with a significant increase in the incidence of fetal congenital malformations.

In humans, hyperglycemia or diabetic associated malformations are commonly found in the cardiovascular system and the neural tube, although virtually any organ or system may be affected (Singer 1995). Caudal regression syndrome, which is characterized by absence of sacrum or coccyx, is almost a specific complication of diabetes mellitus (Rusnak & Driscoll 1965).

The incidence of congenital anomalies has consistently been found to be

higher among diabetic mothers compared to the normal general population. Chung and Myrianthopoulos (1975) from the United States reported that, in a total of 47,000 non-hyperglycemic pregnancies and 947 hyperglycemic pregnancies, 17% of the infants of women with hyperglycemia and 8.4% of those of non-hyperglycemic women had malformations. Soler et al (1976) and Drury et al (1977) studied 701 and 300 hyperglycemic pregnancies respectively. In these two series, malformation rates were 8 % and 6.4% respectively, compared with 1% and 2% in the general population. Again, a similar survey was reported in 1982 (Beard and Lowy 1982). There were 38 (5.72%) cases with fetal congenital anomalies in the established diabetics and 4 (1.79%) in the gestational diabetics. Approximately half of the malformations were classified as major. Overall, the incidence of congenital malformation in hyperglycemic or diabetic mothers is approximately 3 to 4 times higher than that in normoglycemic mothers.

For obvious reasons, human data concerning the incidence of hyperglycemia- induced malformation are mostly based on epidemiological and observational studies. Further studies into the underlying mechanisms of pathogenesis are hindered by ethical constraints. Animal models, both in vivo and in vitro, are therefore developed as an alternative approach.

2.2.2 Animal Studies

Animal studies have also shown that hyperglycemia is associated with various kinds of malformations. The major effects include the induction of malrotation of the tail and abnormal non-closure of the neural tube, i.e., neural tube defect (Pinter et al 1986). In addition, the yolk sac is also affected resulting in a marked reduction in vitelline vessel formation with

sparse, patchy and non-uniformly located visceral yolk sac capillaries. Ultrastructurally, endodermal cells from these visceral yolk sac had a significant reduciton in the numbers of ribosomes, mitochondria and lipid droplets in cytoplasm (Reece et al 1998).

In 1986, Norbert Freinkel described the use of whole rat embryo culture to demonstrate the teratogenicity of hyperglycemia (Freinkel 1986). Whole rat embryos of a strain, Spargue Dawley, were explanted at the stage of organogenesis and cultured in rat sera supplemented with D-glucose of increasing concentrations: 1.5 mg/ml (control), 3mg/ml, 6mg/ml, 9mg/ml and 12mg/ml. This study showed that increasing glucose level in the culture medium resulted in faulty organogenesis of the neural (major lesions) and extraneural (minor leisons) organs in a dose-dependent fashion up to 23% and 49% respectively. Reece et al (1998) reported a similar study using a different rat, the Charles rover, and cultured its conceptuses under increasing hyperglycemic conditions. A dose-related increase in the incidence of malformations was observed with increasing glucose levels: A 20% malformation rate was induced at glucose level approximately twofold (3mg/ml) of normal level (1.5mg/ml), and almost 100% rate at levels greater than six times of control (9.5mg/ml). However, the types of induced malformations did not differ with different glucose concentrations. These data clearly showed that the dysmorphogenic potential of ambient glucose is concentration-dependent although the precise relationships may be quantitatively different in various species or in different strains of the same species.

2.3 Timetables for Embryogenesis: Rats versus Humans

Although hyperglycaemia could induce fetal malformation, such an effect occurs only if the fetus is exposed to the adverse condition during the critical period of organogenesis. In rat, malformations were found in the offspring of streptozocin-induced hyperglycemia (Eriksson et al 1989) and spontaneous diabetic BB/E rats (Shepard et al 1980) that did not receive insulin therapy during gestational day 6-10, which is the early period of organogenesis when the basic tissues are formed. This early insult exerts profound effects on subsequent development during the remaining period of pregnancy.

In humans, a normal gestation lasts 280 days from the start of the last menstral period, or 266 days from the time of conception. In rats, the total gestational period takes 21 days on average only depending on strains. It is therefore not surprising that the periods of organogenesis, when embryos are most vulnerable to the effects of teratogens, are different between humans and rats. The understanding of the difference is very important if any observations made in experiments using rat embryos are to be extrapolated to human embryos.

Comparative timetables in rat and man for embryogenesis are as follows: In rats, implantation occurs on day 6-7 of pregnancy, the neural plate is established on day 9.5, the anterior neuropore closes on day 10.5 and closure of the posterior neuropore occurs on day 11.5. In humans, establishment of the neural plate takes place on day 18-20, the anterior neuropore is closed on day 24-25 and the closure of posterior neuropore occurs on day 26-27. In rats, formations of body organs occur between gestational day 6-10 which is equivalent to 3-6 weeks after conception in human cases (Shepard et al 1980; Mills et al 1989).

2.4 Mechanisms of Hyperglycemia Induced Teratogenesis

Many factors have been explored and reported to be associated with hyperglycemia-induced teratogencity, including deficiency in arachidonic acid (Goldman et al 1986; Pinter et al 1988), myo-inositol (Hod et al 1986; Sussman & Matschinisky 1988; Weigensberg et al 1990; Bruce & Bray 1983; Greence et al 1987), as well as genetic factors (Eriksson et al 1986, 1988). Even though Eriksson has attempted to propose a unifying concept for hyperglycemia-induced teratogenesis (Eriksson et al 1996), the precise mechanisms are still unknown. Recently, there are increasing evidences that oxidative stress may play a vital and central part of teratogenesis.

2.4.1 What are Free Radicals?

Electrons in atoms occupy regions of space known as orbitals. Each orbital can hold a maximum of 2 electrons, spinning in opposite directions. A free radical can be simply defined as any species capable of independent existence that contains one or more unpaired electrons: an unpaired electron being one that is alone in an orbital. Most biological molecules are non-radicals, containing only paired electrons. Because electrons are more stable when paired together in orbitals, free radicals are, in general, more reactive than non-radical species, although there is a considerable variation in their individual reactivity (Halliwell et al 1989). In general, most of the free radical species in biological systems are derived from oxygen. The most important electron acceptor in the biosphere is molecular oxygen, which, by virtue of its bi-radical nature, readily accepts unpaired electrons to give rise to a series of partially reduced species collectively known as 'reactive oxygen species' or 'ROS'.

Free radical reactions have been implicated in the pathology of more than

50 human diseases (Halliwell et al 1991). Radicals and other reactive oxygen species are formed constantly in the human body, both by deliberate synthesis and by chemical side-reactions occurring when antioxidant defenses are inadequate. Target oxidations, also known as oxidative stress, are mediated by increase in the generation of free radicals or by decrease in the protective ability of the body to withstand normal levels of peroxidation, and can damage lipids, proteins, carbohydrates and DNA. Oxidative stress plays an important role in hyperglycemia, which is associated with increased production of free radicals.

Free radicals can react with other molecules in a number of ways. Thus, if 2 radicals meet, they can combine their unpaired electrons and join to form a covalent bond- a shared pair of electrons. However, if a radical gives one electron to, takes one electron from or simply joins on by addition to a non-radical, that non-radical becomes a radical. Thus, a feature of reactions of free radicals with non-radicals, which includes most biological molecules, is that they tend to proceed as chain reactions: One radical generates another and so on. Only when two free radicals meet can these chain reactions be terminated (Halliwell et al 1991).

2.4.2 Major Free Radical Species Involved in Hyperglycemic Teratogenesis

Major reactive oxygen species of interest include the hydroxyl radical ($\cdot OH$), superoxide (O_2^-), hydrogen peroxide (H_2O_2), peroxyl radical (ROO^-) and singlet oxygen ($^1O_2$), all of which are also capable of causing biomolecular damages (Warso et al 1983).

Among the various reactive oxygen species, the hydroxyl radical, singlet oxygen and the peroxyl radical are the most potent and highly damaging.

The hydroxyl radical attacks neighbouring biomolecules at very high rates. However, the half-life of hydroxyl radicals in biological system is rather low, in the order of 10^{-9} second. The half-life of singlet oxygen is in microseconds and that of peroxyl radicals is in seconds. Besides these, there are also several secondary radicals or excited states derived from the primary species (Devasagayam et al 1996).

2.4.3 Molecular Damage Induced by Reactive Oxygen Species

Normally, cells are protected against free radicals by the action of naturally occurring antioxidants. In the event of oxidative stress, free radicals are in excess, which will lead to the following cellular damages.

DNA Damage - The primary site of oxidative damage is DNA. Leading to altered bases and strand breaks. Both types of change, if not repaired, affect the cell structure and function (Devasagayam et al 1996).

Protein Damage - The reactive oxygen species can induce significant structural alternations in proteins through cross-linking and fragmentation (Chang et al 1985), which may lead to inactivation of proteins (Devasagayam et al 1996).

Lipid Damage - Cellular membranes contains polyunsaturated fatty acids which possess large amount of double bonds and are therefore one of the major targets of free radicals. Oxidative damage results in lipid peroxidation, which is a chain reaction involving initiation, propagation, and termination reactions. Unchecked peroxidative decomposition of membrane lipids is catastrophic for living systems, leading to adverse alterations in other cellular molecules, either directly or indirectly through byproducts (Devasagayam et al 1996). Lipid peroxidation also reduces the

synthesis of prostacyclin and ultimately lead to an imbalance between prostacyclin and prostaglandin (Warso et al 1983). This prostanoid imbalance could cause indirect tissue injury and maldevelopment.

2.4.4 Supporting Evidence of Reactive Oxygen Species Causing Anomalies

Recently, there are many clinical and experimental studies which supported the critical role of over-production of free radicals in the pathogenesis of hyperglycemia-induced embryopathy. Abnormal GSH (one of the primary cellular antioxidants) metabolism and therefore a reduced ability to degrade free oxygen radical were found in embryos exposed to hyperglycemic environment (Hales & Brown 1991). Significant reduction in the incidence of hyperglycemia-induced malformation were observed if embryos were observed when supplemented with a wide variety of free oxygen radical scavengers, including superoxidase, catalase, glutathione peroxidase and vitamin E (Eriksson et al 1989; 1991; 1993; Sivan et al 1996). Free radical scavengers or antioxidants are enzymes or molecules that prevent cellular damages by degrading free radicals. Similarly, Hagay et al (1995) demonstrated a significant lower rate of malformation in transgenic hyperglycemic embryos which over-expressed superoxide dismutase, which is an extremely potent antioxidant.

Wentzel et al (1999) in their in vitro and in vivo study found an increased concentration of F_2-isoprostane compounds in conceptuses of hyperglycemic pregnant rats representing a higher free oxygen radical activity. F_2-isoprostane is the most stable and reliable marker so far for the estimation of the degree of lipid peroxidation caused by free radicals (Roberts & Morrow 1994; Awad et al 1996).

2.4.5 Hyperglycemia and formation of Free Radicals

Hyperglycemia induces changes in many biochemical pathways which contribute to the increase in free radical production either directly, or indirectly due to a reduction in antioxidative capability. Glucose may undergo autoxidation which describes the capability of glucose to enolize and thereby reduce molecular oxygen and yield oxidizing intermediates (Hunt et al 1988; Wolff et al 1987; Jiang et al 1990).

The influx of glucose into cells stimulates the polyol pathway (Hohman & Beg 1994), which results in a reduced NADPH to $NADP^+$ ratio and an increase in the cytosolic NADH- to -NAD+ ratio (Williamson et al 1993). As a consequence, activity of the glutathione redox cycle, a principal biological antioxidative pathway which is NADPH-dependent, is reduced (Asahina et al 1995). On the other hand, free radicals are generated via an increased synthesis of prostaglandin H_2 from prostaglandin G_2 due to an overactivity of the converting enzyme hydroperoxidase which uses NADH as a cofactor (Smith 1986).

There is evidence that hyperglycemia itself may depress natural antioxidant defenses, such as superoxide dismutase (Oda et al 1994).

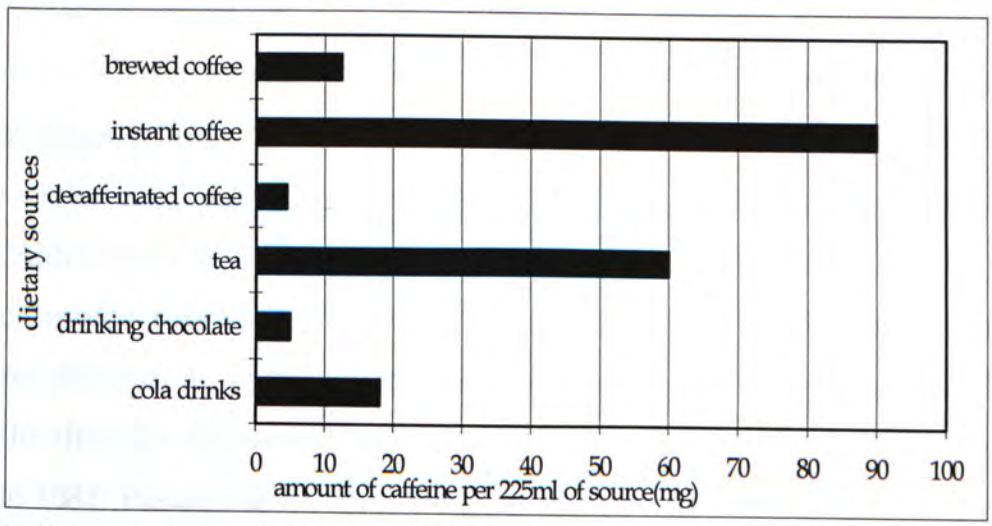
Hyperglycemia also cause cumulative irreversible alterations in stable macromolecules, including the excessive formation of glycosylated proteins (Brownlee et al 1984). It has been shown that hyperglycemia increased gylcosylation of erythrocyte superoxide dismutase, resulted in a reduction in its antioxidative ability (Arai et al 1987). Glycosylation products themselves may be oxidized under catalysis by transition metals to form autoxidation products i.e., free radicals. (Ahmed et al 1998, Baynes et al 1991; Jiang et al 1990).

Chapter 3: Caffeine as Teratogen and Antioxidant

3.1 Popularity of Caffeine

Caffeine ($C_8H_{10}N_4O_2$) is one of a family of methylated xanthines, often referred to as 1,3,7-trimethylxanthine or merely xanthine.

Humans possess an extraordinary propensity to consume psychoactive compounds. Of the numerous substances used, none is more popular than caffeine. More than 80% of the world's population, irrespective of age, gender, geography, or culture, consume caffeine daily (ICO 1991). The average per capita daily intake of caffeine varies between 200 mg to 400 mg, which is equivalent to 2 to 6 cups of coffee or tea (Gilbert 1984). The figure below shows the approximate caffeine content in each 225 ml of different dietary sources (Bunker & McWilliams 1979; Burg 1975).



Caffeine content of the main dietary sources

3.2 Basic Metabolism of Caffeine

Following ingestion, caffeine is readily absorbed and distributed throughout the entire body. Both animal and human studies have shown that there are no significant physiological barriers limiting the passage of caffeine through any biological membranes (Arnaud 1984; 1983; 1987; 1988; Bonati et al 1984), including the human placenta (Goldstein & Warren 1962). Caffeine concentrations in blood are highly correlated with those found in breast milk (Bailey et al 1982; Berlin 1981), amniotic fluid and fetal tissue (Brazier et al 1983; Van't Hoff 1982). Biotransformation of caffeine is regulated by cytochrome P-450 enzyme system in the liver. In adults, caffeine is completely transformed, with less than 2% of the ingested compound being recoverable in urine unchanged.

The half-life of caffeine ranges from 0.7 to 1.2 hours in rodents, and 2.5 to 6 hours in humans (Blanchard & Sawers 1983; Bonati et al 1982). A number of factors affect the rate of elimination of caffeine. Newborns show a markedly decreased rate of clearance due to the immaturity of hepatic systems, namely cytochrome P-450 (Aldridge et al 1979; Aranda et al 1979; Carrier et al 1988) and some demethylation and acetylation pathways (Aldridge et al 1979; Aranda et al 1979; Pariente-Khayat et al 1991). The clearance rate for caffeine decreases during the course of pregnancy, resulting in an approximate threefold increase in the plasma half-life of the drug by the second and third trimester (Aldridge et al 1981; Knutti et al 1981; Parsons & Pelletier 1982). Cigarette smoking increases the rate of caffeine elimination (Joeres et al 1988).

3.3 Biological Actions of Caffeine

The primary biological action of caffeine is the stimulation of the central nervous system. Like other methylxanthines, this effect is mediated by blocking the effects of the neuromodulator adenosine (Milon et al 1988; Hirsh 1984; Spindel 1984). Caffeine and other methylxanthines affect the contractility of the heart and blood vessels by influencing neurotransmission in the central nervous system and in the peripheral nervous system (Fredholm 1984; Daly 1993; Nygard et al 1997). In the gastrointestinal system, caffeine stimulates pancreatic hormone secretion, gastric secretion and gall bladder contraction (Fredholm 1984). Caffeine also stimulates respiration and causes relaxation of tracheal smooth muscle by increasing the effect of acetylcholine or cholinesterase inhibitors (Fredholm 1984). Lastly, the diuretic effect of methylxanthines could be explained by the increase in renal blood flow and glomerular filtration rate caused by the chemical itself.

3.4 Teratogenicity of Caffeine

3.4.1 Animal Studies

3.4.1.1 Teratogenic Effects of Caffeine in Animals

Nishimura et al (1960) was one of the earliest reports demonstrating teratogenic effect of caffeine in vivo. They found a malformation rate of 18% to 43% in one hundred SMA mice given one single dose of 250mg/kg of caffeine between days 10 to 14 of pregnancy. This observation was subsequently confirmed by other experiments on mice (Knoche & Konig 1964; Fujii et al 1969; Snigorska & Bartel 1970; Bartel & Gnacikowska 1972; Elmazar et al 1981; 1982; Scott 1983). Fujii et al (1969) also found that a single, high daily injection of caffeine resulted in malformations in 18% to 21% of fetuses, compared with only 6% if the same amount was given in divided doses. No teratogenic effect however was found when a lower dose of up to 36 mg of caffeine /kg on Days 6-15 of gestation was given (Food and Drug Research Labs 1973).

Similar in vivo experiments in the Osborne-Mendel rats and Wistar rats showed that maternal caffeine exposure up to 125mg/kg caffeine was associated with a higher incidence of fetal malformation including ectrodactyly and delayed skeletal ossification (Bertrand et al 1970, Collins et al 1981, Ikeda et al 1982; Smith et al 1987). Using Spargue Dawley rats, Fujii & Nishimura (1972) found that the incidence of fetal malformation is higher with daily caffeine consumption of 300 mg/kg, but not with 180 mg of caffeine/kg/day.

Using an in vitro whole rat embryo culture model, Iwase et al (1994) was able to demonstrate a dose-dependent effect of caffeine on the incidence of

fetal malformation. In 1997, Marret et al reported their work of culturing mouse embryos with caffeine in concentrations equivalent to heavy caffeine consumers. They found that a short exposure to caffeine induces significant disturbances of early fetal neurogenesis including failure of neural tube closure, excessive proliferation of neuroepithelial cells and premature evagination of telencephalic vesicles.

In summary, animal studies showed that malformations associated with caffeine consumption included craniofacial anomalies (labial or palatal clefts), ectrodactyly (absence of toes), and abnormalities of the limbs and digits (Aliverti et al 1979; Battig & Sullivan 1987; Collins 1979; Collins et al 1981; Elmazar et al 1981; Fujii 1976; Fujii & Nishimura 1969; Nash & Persaud 1988; Purves & Sullivan 1993; Smith et al 1987). More rarely observed are malformations of the eyes, the cranium, and the tail (Nolen 1988; Wilson & Scott 1984) as well as cardiac malformations (Mulhivill 1973). One effect of caffeine consistently demonstrated by most investigations is a delay in skeletal ossification (Collins et al 1981; 1983; Elmazar et al 1982; Fujii & Nishimura 1972; Nolen 1981; 1982; Palm et al 1978). With respect to brain development, three types of disorders in neurulation are observed, including thickening of the neuroepithelium, premature evagination of the ventral prosencephalic neuroepithelium , and absence of neural tube closure (Marret et al 1997).

3.4.1.2 Teratogenic Dose of Caffeine

Most experimental studies so far supported that caffeine is teratogenic, but only at a relatively high dose. Thus, caffeine can be considered a weak teratogenic agent. Teratogens may be classified as " peak blood level" type or "area under the curve" type when the blood level is plotted against time. Caffeine may be considered a teratogen of the " peak blood level"

type, and a level of 60-80 μg/ml or more in plasma is required for teratogenicity (Elmazar et al 1982; Ikeda et al 1982; Smith et al 1987; Collins 1979). Plasma caffeine lower than 60μg/ml, although not teratogenic, may still adversely affect the fetus resulting in fetal loss (Gilbert et al 1973), growth retardation or delayed skeletal ossification (Purves et al 1993).

Administration of 80mg/kg of caffeine per day to pregnant rats in their drinking water resulted in a mean peak plasma caffeine level of 5.7μg/ml (Ikeda et al, 1982). In order to reach the teratogenic threshold level of 60 μg/ml, a rat has to consume 800 mg (80 × 10) of caffeine a day. Using these hypothetical data, 800mg/ day caffeine is equivalent to a rat (weighing 10 kg) consuming 8,000 mg (800 × 10) of caffeine a day, or 80 cups of coffee, 200 cups of tea or, 228 cans of cola.

In human, the administration of 91mg of caffeine per day resulted in a mean peak plasma caffeine level of 1.01μg/ml (Hasegawa et al 1989). Hence, in order to reach the teratogenic threshold level of 60μg/ml in rat, one has to consume 5460 mg (91 × 60) of caffeine a day, which is equivalent to 55 cups of coffee, 137 cups of tea, or 156 cans of cola.

3.4.1.3 Interspecies Sensitivity

The sensitivity of different animal species to teratogenic effects is variable, with mice being more sensitive than rats. In mice, the malformations appear at doses of 50-75 mg/kg (Wilson & Scott. 1984) while in rats, the events happen at doses of 80-100 mg/kg (Collins 1979; James 1991; Purves & Sullivan 1993). Indeed, the frequency of fetal malformations differs from one strain of rats to another. For example, the Sprague Dawley strain is more liable to maldevelopment than the Wistar strain when exposed to

teratogens.

3.4.2 Human Studies

Although there is strong evidence that high dose caffeine is teratogenic to animal embryos, whether such effects exist in human gestation is still unknown. Due to ethical constraints, human data are mostly derived from epidemiological and observational studies, amongst which methodological problems such as inadequate sample size, selection bias, recall bias or the presence of uncontrolled confounding factors commonly existed.

Borlee et al (1978) reported a significant rise in incidence of congenital malformations in women who drank more than 8 cups of coffee per day, although potential confounding effect of and interaction between other teratogens such as tobacco and nicotine were not taken into account. Jabsoson et al (1981) reported three subjects with ectrodactyly and other malformations born to mothers who drank between 8 to 25 cups of coffee per day, which was equivalent to 1100 mg to 1777 mg of caffeine per day, (Jabsoson et al 1981). More recently, Furuhashi et al (1985) found that there was a significantly higher risk of congenital abnormalities in individuals who consumed more than 5 cups of coffee per day when compare with those who did not.

On the other hand, many studies have failed to demonstrate any relationships between congenital malformations and caffeine consumption (Heinonen et al 1977; Kurppa et al 1983; Lechat et al 1980; Linn et al 1982; Martin et al 1982; McDonald et al 1992; Morris et al 1981; Nelson et al 1971; Olsen et al 1991; Roseberg et al 1982; Tikkanen et al 1988).

In summary, the relationship between caffeine and fetal malformation is far from clear. Even if exist, such an effect is likely to occur only in very high doses of caffeine consumption.

3.5 Possible Mechanisms for the Teratogenic Actions of Caffeine

Caffeine at high plasma concentration of 300 may disturb intra-cellular signaling by stimulating the release of calcium from intracellular stores (Tsai & Barish 1995). At concentration of 100 μg/ml, caffeine increases the level of cyclic adenosine monophosphate (cAMP) (Nehlig et al 1992), which was though to be important in inducing digit malformations and hypoplasy of limbs (Fujii & Nishumura 1969), and in cardiac malformations (Imamura et al 1992). High cAMP levels may also dilate peripheral blood vessels, which is thought to induce hyperemia of the tail and hematoma in the yolk sac (Iwase et al 1994). High caffeine concentration has also been found to induce placenta injury (Schreiner et al 1986), reduction in intervillous placental blood flow (Kirkinen et al 1983) and increased concentration of noradrenaline, all of which might lead to abnormal embryonic development (Kirkinen et al 1983).

However, such high concentration of is unlikely to be achievable after ordinary consumption of caffeine in human. The plasma concentrations associated with typical patterns of consumption usually do not exceed 10 μg/ml, usually between 0.5 and 3.0 μg/ml (Fredholm 1985). Therefore the most plausible principal mechanism of action, is by competitive blockade of adenosine receptors (Fredholm 1995) due to the similar molecular structure between caffeine and adenosine. As adenosine generally functions to inhibit physiological activity, the blockade of adenosine receptors by caffeine has broadly stimulant effects (Hughes et al 1991).

Caffeine was also thought to induce its teratogenicity through increased release of corticosterone. Labial and palatal clefts have been produced in mice by glucocorticords, and a dose of 100 mg/kg of caffeine increases the concentration of corticosterone in mice by tenfold (Elamzar et al 1981).

3.6 Caffeine as an Antioxidant

Apart from acting as a teratogen, caffeine has been also known for its potential beneficial antioxidative power. As early as the 1970s, caffeine had been shown to exert protective effects against oxic component of radiation damage in cell culture systems (Kesavan & Natarajan 1985), plant systems (Kesavan et al, 1973), and in aqueous suspensions of bacterial spores (Kesavan & Powers 1985; Raghu & Kesavan 1986). It was later shown that the radioprotective effect of caffeine was due to its ability to scavenge potentially damaging hydroxyl radical and electrons (Farooqi & Kesavan 1992; Raghu & Kesavan 1986), thereby protecting cells against apoptosis induced by free oxygen radicals released by radiation (Zhen & Vaughan 1995).

In 1991, Shi reported on the reaction of caffeine with hydroxyl radicals. They found that caffeine effectively scavenges hydroxyl radicals with a constant reaction rate comparable to those of other efficient hydroxyl radical scavengers, and such behavior is dose-dependent. In 1996, Devasagayam et al carried out a detailed study on the effect of caffeine on lipid peroxidation and they found that caffeine was a more potent antioxidant than glutathione and ascorbic acid - two well-established antioxidants.

Mechanisms of action of antioxidants include removal of oxygen, scavenging reactive species, inhibition of reactive species formation, as well as upregulation of endogenous antioxidant defenses (Halliwell 1990). As for caffeine, its antioxidant action is exerted through scavenging reactive species produced in oxidation of substrate (Raghu & Kesavan 1986, Shi & Dalal 1991; Devasagayam et al 1996).

3.7 Combined Effects of Caffeine and Other Substances

Combined effects of caffeine and common teratogens are widely known (Mulvihill 1973; Collins et al 1979; 1981). Recent studies on chicken embryos and rodents have proven that caffeine does potentiate the teratogenic effects of many substances, including nicotine (Gilani & Persuad 1986; Nash & Persuad 1988; 1989), irradiation (Kusama et al, 1989; Muller et al 1989; 1983; 1985), alkalizing agents (Fujii & Nakatsuda 1983; Nakazawa et al 1985) and ethanol (Gilani & Persuad 1985; 1986; Henderson et al 1991; Ross & Persuad 1984; 1989; 1990; Tanaka et al 1985); only two studies did not demonstrate this phenomenon (Fadel & Persuad 1991; 1992).

However, to our knowledge, there is no data on the possible combined effects of caffeine and hyperglycemia. We believe that it is clinically important to investigate the interaction between these two since it is not rare to encounter a hyperglycemic mother who is also a caffeine drinker.

Chapter 4: Combined Effects of Hyperglycemia and Caffeine on Early Embryogenesis- A Question to be Answered

4.1 Possible Links between Hyperglycemia and Caffeine

Diabetes mellitus/ Hyperglycemia is a common disease worldwide and is the most common cause of fetal malformation due to maternal disease (Malins 1979; Molsted-Pedersen & Pedersen 1985). Since over 80% of the population consumes caffeine daily (ICO 1991), it would not be rare to encounter a pregnant diabetes mellitus patient who also takes caffeine. What then would be the effect of caffeine consumption on hyperglycemia induced teratogenesis?

It is possible that caffeine, being a teratogen itself at high concentration, may directly potentiate the teratogenic effects of hyperglycemia. Furthermore, caffeine is known to increase blood glucose level after consumption in a few hours in a dose dependent fashion (Cherskin 1968), which might indirectly enhance the teratogenic effect of hyperglycemia.

However, their interaction may be a beneficial one. The antioxidative effect of caffeine may ameliorate the teratogenic actions of hyperglycemia which was mediated through the production of reactive oxygen species (Sivan et al 1996; Eriksson et al 1989; 1993; 1991; Wentzel et al 1999).

So far, the combined effects of caffeine and hyperglycemia on embryonic development have not been studied. We postulate that caffeine in low dose may prevent hyperglycemia-induced malformations by reducing oxidative stress. At high dose, however, the teratogenic effects of caffeine are so profound that it outweighs the beneficial antioxidant effect.

4.2 Objectives of the Present Study

The main research objectives of the present study are:

(i) To establish an animal model for the study of hyperglycemia induced fetal malformation.

(ii) To reconfirm that hyperglycemia results in oxidative stress, which is in turn associated with a higher incidence of malformations.

(iii) To quantify the teratogenic dose of caffeine

(iv) To assess the effect of sub-teratogenic level of caffeine on the incidence of malformation and on the level of oxidative stress among cultured embryos exposed to teratogenic level of glucose.

4.3 Hypothesis

There are two main hypotheses to be tested.

(i) Addition of a sub-teratogenic concentration of caffeine reduces/ abolishes the incidence of malformation caused by hyperglycemia.

(ii) Addition of a sub-teratogenic concentration of caffeine reduces/ abolishes oxidative stress caused by hyperglycemia.

The null hypotheses are therefore,

(i) $\mu_1-\mu_2 = 0$. Where μ_1 is the mean morphology score in embryos after in vitro culture in teratogenic doses of glucose, and μ_2 is the mean morphology scores of embryos after in vitro culture in teratogenic doses of glucose and sub-teratogenic doses of caffeine.

(ii) $\mu_3-\mu_4 = 0$. Where μ_3 is the mean 8-isoprostaglandins F2α level in embryos after in vitro culture in teratogenic doses of glucose. And μ_4 is the mean 8-isoprostaglandins F2α level in embryos after in vitro culture in teratogenic doses of glucose and sub-teratogenic doses of caffeine.

Section II: Research Designs and Methods

Chapter 5: Materials and Methods

5.1 Licenses

The use of animals was approved by the Animal Research Ethics Committee of the Chinese University of Hong Kong (Reference Number 99/011/MIS), and the project was carried out under personal license (Reference Number (96) in DHNTE 007/5 Pt. 20), accepted by the Department of Health, Hong Kong.

5.2 Overall Study Design

This was an experimental study using a whole rat embryo culture model. Explanted rat embryos were culture under different concentrations of glucose and caffeine. The effects of different combinations of teratogens on the overall embryonic development were assessed by morphological scoring and the effect on free radical activities were measured biochemcially. The basis for choosing this model and methods will be discussed in next Chapter.

5.2.1 Whole Embryo Culture Model

The whole embryo culture system was purposely established for the present project. It took the candidate more than 6 months to optimize the system and the following was the final protocols used for all experiments reported in this thesis.

5.2.1.1 Animals

Rats of an outbred strain of Sprague Dawley (SD) at gestational day 9.5 were supplied by the Animal House of the Institute as the embryo donors for this study. Embryonic age was calculated from 00.00h on the night of mating. After timed-mating, pregnant rats were caged in groups of four with other females at the same stage of pregnancy until they were required for experimentation. To minimize environmental variance, all rats were fed a commercial guinea pig diet (Lab Diet, USA) and had access to food and tap water ad libitum. They were kept at an ambient temperature of 22°C with a constant 9-hour light and 15-hour dark cycle. A total of 811 embryos, removed from 101 pregnant rats, were used for the generation of the final data presented in this thesis.

5.2.1.2 Explantation of Embryos and Serum Collection

Pregnant rats were taken between 09.00 to 10.00 h on day 10 of gestation (i.e. 9.5 day old) and deeply anaesthetized with diethyl ether (Merck, Germany). Maintenance of anaesthesia was achieved by the use of a nose-cone. A liberal volume of 70% methanol (Merck, Germany) was poured over the abdominal wall of the rats to prevent fur interfering with the subsequent dissection and to also assist in maintaining a clean surgical field. The abdominal wall was gripped with toothed forceps and cutting were performed from the suprapubic area to the lateral costal margins. The resulting abdominal wall flap was reflected onto the thorax and the gut were pushed the right side of the abdominal cavity to exposure the retroperitoneal structures. The aorta was cleared from surrounding fat and connective tissue from the level of the renal arteries to just below the aortic bifurcation. Blood sample was taken using a 21G needle and syringe (DA Medicrat, Korea) at the aortic bifurcation. After blood withdrawal, uteri were removed from the rat and placed in a Petri dish containing Hank's balanced salt solution (Gibco BRL, USA) at room temperature. The heart of the rat was cut to ensure death and the animal was disposed according to our laboratory regulation.

The explantation of conceptus was performed in Hank's Balanced Salt Solution at room temperature. Under a dissecting microscope (Olympus, Japan), with the use of ultra-fine forceps (Dumont, USA), the uterine walls were torn open and the decidua was separated from the conceptus and discarded. The Reichert membrane of each conceptus, together with its internally adherent parietal endoderm was opened along its length and stripped back to the ectoplacental cone. The embryos, with the amnion, visceral yolk sac and ectoplacental cone intact, were placed into culture

medium within 2 hours after the start of anaesthesia of the mother rats. In order to minimize variations, only embryos with crown rump length of 1.5 ± 0.3 mm were used for the experiments

5.2.1.3 Preparation of serum

After collection, blood samples were immediately centrifuged (MSE, England) for 10 minutes at 3500 revolutions per minute to clear the plasma fraction of cells. The cell-free plasma was allowed to clot at room temperature and the clot thoroughly squeezed with forceps to release the serum. The tubes were then recentrifuged for 10 minutes at 3500 revolutions per minute to separate the blood cells again, together with the collapsed clot. The clear serum supernatant was then decanted and pooled. The pooled serum was then heat-inactivated for 30 minutes at 56°C in water bath (Memmert, Germany) and either used immediately or stored at -20°C before use in culture.

5.2.1.4 Culture Media

The standard culture medium for each embryo was a mixture of 0.5 ml of rat serum and an 0.5 ml of Dulbecco's Modified Eagles Medium (DMEM) (Gibco BRL, USA). Penicillin G (Sigma, UK) and streptomycin sulfate (Sigma, UK) were added to culture media to final concentrations of 60μg/ml and 100 μg/ml media, respectively.

5.2.1.5 Embryo Culture

Explants were cultured at 37°C in glass vessels attached to a rotating-bottle culture unit (BTC Engineering, UK) housed within a

precision incubator (BTC Engineering, UK). The period of culture was 48 hours. A maximum of 4 embryos with corresponding culture medium could be placed in 1 culture vessel. The culture vessels were rotated at a rate of approximately 60 revolutions per minute and were continuously aerated with 5% CO_2; 5% O_2 in 90% N_2 for the first 24 hours of the experiment, 5% CO_2; 20% O_2 in 75% N_2 for the next 8 hours and 5% CO_2; 40% O_2 in 55% N_2 for the remaining culture period. The switching of aerating gas was performed automatically by a timer-controlled system designed and assembled by the Engineering Department of the same institution. Gas mixtures were premixed commercially (Hong Kong Oxygen and Acetylene Co. Ltd., Hong Kong).

5.2.2 Experimental Groups

The present study design involves subjects of three major groups.

The first major group is the hyperglycemic group. Embryos within this group were further subdivided into smaller groups exposed to varying degree of hyperglycemic environment to demonstrate the dose-dependency of teratogencity of hyperglycemia. Embryos were randomly assigned to one of four study subgroups. Group 1 was the control group with a normal glucose concentration of 6.7 mmol/l in the culture medium (1.2mg/ml) (Freinkel et al 1986). Embryos in Groups 2, 3 and 4 were exposed to hyperglycemic environments with glucose concentration double (13.3 mmol/l), 4 times (26.7 mmol/l) and 8 times (53.4 mmol/l) of the normal level, respectively.

The second group is the caffeine group. Again, various subgroups were designed to test the effects of increasing concentrations of caffeine as well as quantify the threshold teratogenic dose of caffeine. Again, embryos

were randomly assigned to one of the following groups: group 1, the control group with standard culture medium. Groups 2 to 6 were exposed to 10.0, 20.0. 30.0, 60.0 ,and 90.0 μg/ml of caffeine respectively.

In the last major group, combined effects of hyperglycemia and caffeine were tested. Different combined concentrations of caffeine and hyperglycemia were used for the testing. The subgroups were divided, based on the data obtained from hyperglycemic groups and the caffeine groups: Group 1 was the control group with standard culture medium. Groups 2-6 contained glucose at four-folded hyperglycemic level (13.3mmol/l). Groups 3 to 6 also contained 1, 2.5, 5 and 10 μg/ml of caffeine respectively.

During each experiment, embryos harvested from all rats were mixed together and randomly assigned to one of the experimental groups. This was to avoid the potential errors and variations due to differences between embryos from different subjects.

5.2.3 Morphological Assessment

Embryos were examined after 48 hours of culture at the equivalent of 11.5 days of gestation, by a researcher who was not aware of the study group assignment. Mean yolk sac diameter and crown-rump length were measured. Embryonic morphologies were studied according to a standard morphological scoring system (Fabry et al, 1990), which gives a numerical score to 17 morphological features depending on their stage of development as followed:

Yolk Sac Circulatory system

A score of 1 was given when there was no visible or scattered blood vessels on the yolk sac. A score of 2 was given to those with a corona of blood islands. A score of 3 was given when there were few yolk sac vessels on the yolk sac. Score 3 refers to the full plexus of yolk sac vessels with origins of vitelline artery and vitelline vein still widely separated. A score of 4 was given when the origins of the two vitelline vessels migrated closer to each other. A score of 5 was given when two vitelline vessels separated from each other distally and yolk stalk became narrow and obliterated.

Allantois

When the allantois was free in the exocoelom, a zero score was given. When the allantois fused with chorion, and, at the same time, the chorion showed no visible organization, a score of 1 was given. A score of 2 was given to those where rudiments of two vessels appeared in the allantois as an elongated condensation and showed no circulation. A score of 3 was given to those with umbilical circulation just established. A score of 4 was given when separate aortic origins of umbilical and vitelline vessels were visible.

Flexion

A score of 0 was given to those with ventral convex flexion. A score of 1 meant embryos with posterior end rotation. A score of 2 meant embryos with head and tail folds. A score of 3 was achieved when there was mid-trunk region rotation. Scores of 4 and 5 refered to dorsal c-shaped convex flexion and dorsal convex with spiral torsion, respectively.

Heart

A score of 0 was given when the endocardial rudiment was not visible. A

score of 1 meant the heart rudiment was visible as a horsehoe-like thickening of mesoderm surrounding the front end of the embryo but no heart beat was noticed. A score of 2 meant there was a beating s-shaped cardiac tube. A score of 3 referred to convoluted cardiac tubes. Scores of 4 and 5 were hearts with a 3-chamber appearance and 4-chamber appearance, respectively.

Caudal neural tube

Flat neural plates were attributed to a zero score. A score of 1 was given for a closing but unfused neural fold. Scores 2 and 3 were given to fused neural folds and opened, but formed, posterior neuropore. Scores of 4 and 5 were closing and closed posterior neuropores, respectively.

Hindbrain

Again, a flat neural plate gained a zero score, but if neural folds had a v-shape in tangential view, they scored 1. A score of 2 was given when the edges of the monoencephalic folds were closer to each other and had a u-shape in the tangential view. A score of 3 meant part of the folds had fused and the anterior neuropore was formed but open. Scores of 4 and 5 were for those with anterior neuropore closed but rhomencephalon formed, and for pronounced pontine flexion with transparent roof of 4th ventricle, respectively.

Midbrain

A flat neural plate gained a zero score. A score of 1 was given to proencephalic brain folds with a v-shape in tangential view. A score of 2 was given to those where the edges of the prosencephalic folds were closer to one other and had a u-shape in tangential view, but no fusion of folds had occurred in any part of the prosencephalon. A score of 3 was given for partially fused prosencephalic folds. A score of 4 was given to a

completely fused prosencephalon and the embryos presented a rams head when viewed laterally. Scores of 5 and 6 were given if the embryo presented terlencephalic evaginations in the front view with fissura telencephalia in the lateral view, and for well elevated telencephalic hemispheres, respectively.

Forebrain

A flat neural tube scored 0. A score of 1 represented neural folds which had a v-shape in tangential view. A score of 2 was given when the edges of the mesencephalic folds were closer to each other and had a u-shape in the tangential view, whilst no fusion of the folds had occurred in any part of the mesencephalon. Scores of 3 and 4 represented partially fused and completely fused mesencephalic folds, respectively. A score of 5 was given if the forebrain showed a visible division between mesencephalon and diencephalon.

Otic System

When there was no sign of otic development, a zero score was given. Scores of 1 and 2 were given to flattened or indented otic primordium and otic pit respectively. A score of 3 was given for a closed otic vesicle which was not yet separated from epidermis. When the otic vesicle completely separated from epidermis, a score of 4 was given. A score of 5 and 6 were given to otocyst with dorsal recess and with endolymphatic duct, respectively.

Optic System

When there was no sign of optic development, a zero score was given. Scores of 1 and 2 were given to a sulcus opticus and elongated optic primordium respectively. A score of 3 was given to an ovid optic primordium. A score of 4 was given for a primary optic vesicle with open

optic stalk while a score of 5 was given for an indented lens plate.

Branchial Bars

A score of 0 meant no branchial bar was visible. Scores of 1, 2, 3, and 4 were gained when one, two, three, or four branchial bar(s) was/ were visible.

Forelimb

A score of 0 meant no sign of forelimb development. A score of 1 was given when distinct evagination of the Wolfian crest was visible at the level of somites 9-13. Scores 2, 3 and 4 refered to a fore limb bud, a paddle shaped forelimb bud and a distinct apical ridge on a forelimb bud, respectively.

Hindlimb

A score of 0 meant no sign of hindlimb development. A score of 1 was given when distinct evagination of the Wolfian crest was visible. Scores 2 and 3 refered to a hindlimb bud and a paddle-shaped hindlimb bud, respectively.

Somite

A score of 0 was given when there were 0 to 5 somites countable. Similarly, scores 1, 2, 3, 4 and 5 were given when there were 6 to 10, 11 to 15, 16 to 20, 21 to 25 and 26 to 30 somites countable.

Embryos and yolk sac after examination, and the corresponding culture medium, were frozen at -80°C for further assay.

5.2.4 Quantitation of Oxidative Stress

The concentration of 8-isoprostaglandin F2α in the embryo and yolk sac were measured as indicators of oxidative stress. Before performing this assay, the samples had been snap frozen in liquid nitrogen and stored at -80°C so as to prevent any in vitro generation of 8-isoprostaglandin F2α. Assays were performed within six months of samples collection. The extraction, hydrolysis and purifications methods described by Bligh and Dyer (1959) and Morrow et al (1990) were used to determine the total amount of 8-isoprostane in embryos since most of the 8-isoprostaglandin F2α were esterfied in lipids. Assay of the post-culture culture medium was also performed during the initial phrase of the study but was subsequently abandoned (please refer to Chapters on Results and Discussion for details.)

Each embryo or yolk sac was separately homogenized by a polytron type homogenizer in 1 ml ethanol for isoprostane extraction (Merck, Germany), and the sample was vortexed for 2 minutes, stored at 4°C for 5 minutes, then centrifuged at 3000 revolutions per minute for 10 minutes. After this step, the supernatant containing 8-isoprostane was sent for further processing as described below, while the precipitated protein would be frozen at -20°C until protein assay, which will be discussed in section 5.5. To the supernatant, 1ml of 15% potassium hydroxide (Merck, Germany) was added and the sample was incubated at 40°C for 1 hour. The solution was then diluted to a total of 10ml with Ultra Pure water (Milli Q Plus; Millipore, Sweden) and the pH was lowered below 4.0 with 500µl 5N Hydrochloric acid (Merck, Germany). The sample was passed through a C-18 Reverse Phase Cartridge (Sep-Pak Vac, USA) pre-conditioned by rinsing with 5 ml methanol (Merck, Germany) followed by 5ml Ultra Pure water. The cartridge was rinsed with 5ml Ultra Pure water followed by

5ml of HPLC-grade hexane (BDH, England), and the absorbed 8-isoprostaglandin F2α was subsequently eluted from the column with 5 ml ethyl acetate (Lab Scan, Thailand) containing 1% methanol. The ethyl acetate was then evaporated from the 8-isoprostaglandin F2α samples under nitrogen gas (Hong Kong Oxygen and Acetylene Co. Ltd., Hong Kong). Thereafter, 1 ml of EIA buffer was added to re-dissolve the 8-isoprostaglandin F2α. Amounts of 8-isoprostaglandin F2α were then measured by an EIA Kit (Cayman Chemical Co., Australia). The samples were added to the wash buffer pre-rinsed EIA plate with equal volume of tracer and antiserum. After 18 hours of incubation at room temperature, the plate was washed by wash-buffer five times. And developed by adding 200μl Ellman's reagent shaken with an orbital shaker (Scientific Industries, New York) for 90 minutes in darkness. Finally, the plate was analyzed in a spectrophotometric plate reader (Bio-Tek Instrument, Inc) at 405nm wavelength.

Regarding the plate set up, each 96 well EIA plate contained a minimum of two blanks (B), two non-specific binding wells (NSB), two maximum binding wells (Bo), two quality controls (QC) and an eight point standard curve. Blanks (B) represent background absorbance caused by Ellman's reagent. Ellman's reagent has some measurable absorbance. Thus, the purpose of blanks set in each plate is for setting a background absorbance value which is substracted from the absorbance readings of all the other wells. Non-specific binding (NSB) means non-immunological binding of the tracer to the well. Even in the absence of specific antibody a very small amount of tracer still binds to the well. The non-specific binding is a measure of this low binding. Maximum binding is the maximum amount of the tracer that the antibody can bind in the absence of free analyte while the total activity well is to measure the total enzymatic activity of the acetylcholinesterase-linked tracer. This is analogous to the specific activity

of a radioactive tracer and is achieved by adding an additional 5 µl tracer to the well in the plate-development step. Quality control wells contain known amounts of 8-isoprostane. Every quality control sample passed through the same steps as the ordinary test samples. Quality control wells act as indicators for the accuracy of the experiments. The eight point standard curve includes known amounts of 8-isoprostane in decreasing concentrations: 500 ng/ml, 250 ng/ml, 125 ng/ml, 62.5 ng/ml, 31.3 ng/ml, 15.6 ng/ml, 7.8 ng/ml and 3.9 ng/ml. These act as reference values for the predictions of amounts of 8-isoprostane in test samples. For pipetting reagents, 50 µl 8-Isoprostane antiserum was added to each well except the Total Activity, the Non-Specific Binding, and the Blank wells. 8-Isoprostaglandin F2α Tracer was also added in volumes of 50 µl to each well except the Total Activity and the Blank wells. 50 µl samples or standards were added to sample wells and standard wells accordingly. Thus, the total volume of each well was 150 µl.

The final step was to converting the absorbance values into concentration values. This was achieved by averaging the absorbance readings from the NSB wells and from the Bo wells, and substracting the NSB average from the Bo average to get the corrected Bo or corrected maximum binding. Then, the %B/Bo was calculated for the remaining wells. To do this, the average NSB absorbance was substracted from the standard 1 absorbance and divided by the corrected Bo and multiplied by 100 to obtain %B/Bo, the step was repeated for the remaining standard and all sample wells. %B/Bo for standards was plotted for against 8-Isoprostane concentrations. The %B/Bo value was for each sample and the concentration of each sample determined by identifying the %B/Bo on the standard curve and reading the corresponding values on the x-axis.

5.2.5 Protein Assay

Protein assay was carried out to assess the total protein content of each embryo and yolk sac. The method used is a modification of the technique developed by Lowry (Lowry et al 1951).

1 ml 1M sodium hydroxide (Merck, Germany) was added to each Eppendorf tube containing an embryo or yolk sac which had been isoprostane extracted and/ or morphologically scored. The tube was then incubated for 2 hours at 37°C (Memmert, Germany), and whirlimixed before and after the incubation. 145 µl 3M hydrochloric acid (Merck, Germany) was added and whirlimixed again. Two 0.5 ml aliquots of each sample were pipetted into test tubes. 2.5 ml Folin A solution containing 1 ml 1% copper sulphate solution (Merck, Germany), 1 ml sodium tartrate solution (Sigma, UK) and 100 ml 2% sodium carbonate solution (Merck, Germany) was added and left at room temperature for 20 minutes. 0.25 ml Folin B reagent with Folin Ciocalteu's reagent (Merck, Germany) in water was added, whirlimixed immediately, and incubated at room temperature for 45 minutes. Standards and samples were read on a UV/Vis spectrophotometer at 750 nm.

For the standard curve 10, 20, 30, 40 and 50µl of 5mg BSA (Sigma, USA) were each made up to 1 ml with 1M sodium hydroxide and the same steps were carried out as those performed for the test samples. Graphs were plotted with the protein concentration against the read absorbance at 750nm (Beckman, USA) to give a straight line. The relationship is linear and therefore, the protein content of the dissolved embryo and yolk sac could be calculated.

5.3 Statistical Evaluation

Between group differences were compared using the Kruskal-Wallis tests and Dunnet's post hoc t-tests as appropriate. A value of $p < 0.05$ was considered to denote statistical significance. All analyses were performed using the Statistical Package for Social Sciences for Windows version 10.0 (SPSS Inc, Illinois, USA).

Chapter 6: Laboratory Considerations

6.1 Whole Embryo Culture Model

For obvious ethical and legal reasons, we cannot perform in vitro or in vivo experiments on human embryos. We decided to use an in vitro rather in vivo model because the former approach enabled the total and precise control of the experimental environment so that variation could be minimized. We have therefore adopted the whole embryo culture model as described by New (1978), which enable the culture of rodent conceptus throughout the period of early organogenesis *in vitro*, for the current study. Using this model, the growth and differentiation of the cultured conceptus has been confirmed to be the same as that observed *in utero*. Such systems have been used to investigate the mechanisms of toxicity of many teratogens.

6.1.1 Subjects

Sprague Dawley rats were chosen as embryo donors. The Sprague Dawley strain has been accepted widely as a dependable and general-purpose research animal in virtually all disciplines of biomedical research, including toxicology and pharmacology for its outbred property. Embyos were explanted, cultured and studied between 9.5 to 11.5 days of gestation, which is the critical period for organogenesis.

6.1.2 Time Mating

The present model demands embryos at specific embryonic ages- day 9.5. Thus, the ability to accurately determine the gestational age of the Sprague Dawley embryos is extremely critical. We have adopted a simple and

reliable method to estimate mating and conception: the examination of vaginal plug.

The ovaries, uterus and vagina of female rats go through regular cyclic changes, which last for 4 to 6 days. The changes in the vagina are accompanied by changes in the superficial cells of this organ which are easily recognizable in smears, made by inserting a moistened wire loop a short distance into the vagina and the rubbing it on a glass slide. The slide is then stained and examined microscopically. The oestrus cycle may be divided into 4 stages: The first 12 hours is the pro-oestrus stage in which epithelial cells are the typical cells found in vaginal smears. This phase is followed by the 27-hour oestrus stage. Ovulation, and conception if mated, usually occur during second half of this stage. The typical superficial cells in vaginal smear in this stage are cornified cells. The third stage is the 16-hour metoestrus in which leucocytes together with some epithelial and cornified cells are found on smears. The last phase is the anoestrus stage, or resting stage, and lasts for 53 hours. Leucocytes and some epithelial cells can be seen under microscopic examination of the smears. Rats at this fertile period were placed with a partner for time-mating.

When mating occurrs, thread-like spermatozoa will be found in the vaginal smear, usually in large numbers. Spermatozoa persist in the vagina for some hours after mating, eventually degenerating to a granular residue. Mating usually results in the formation of a firm vaginal plug. Since rats during the ovulatory period is very fertile, mating usually followed by pregnancies. Therefore, the onset of pregnancy was assumed to coincide with the appearance of spermatozoa in a vaginal smear at the correct stage of the oestrus cycle. Gestational age is measured from the time of conception, which marks the beginning of gestational day 1.

6.1.3 Culture Medium

The most reliable medium for the culture of rat embryos was pure rat serum. It makes no difference whether the serum-donating rats are males or pregnant or non-pregnant females. Also, their ages or strain are not a major concern. The most important criteria is that the rats must be healthy. This is by far the best media for explanted embryos, and has been widely used in laboratories throughout the world for both rat and mouse cultures (Cockroft 1991). However, the use of pure rat serum implied that many more rats have to be scarified just for serum donation because it was difficult to recover enough volume of serum from a pregnant rat to support the culture of all embryos harvest from the same rat (since culturing of 1 embryo requires 1 ml of serum). A more economic and acceptable alternative is to use 50% of rat serum with 50% of defined medium, (DMEM- Dulbecco's modified eagle media) composed of gluocose, L-glutamine, pyridoxine hydrochloride and pyruvate. Further dilution with medium gave more variable result (Cockroft 1991). To minimise the use of animal, I have therefore decided to use a culture mixture of equal parts of serum and medium.

It is known that immediate-centrifuged sera support better growth than delayed-centrifuged sera. The harmful properties of delayed centrifuged serum are probably due to products of normal clotting process, although the precise mechanism is still unknown (Steele & New 1974, Klein et al 1978).

Heat-inactivation of rat serum at 56°C for 30 minutes is an important step. It improves its capacity to support embryonic growth. Total protein synthesis by the embryo is increased and the frequency of abnormalities of the heart, neural tube, allantois and embryo posture is reduced (Steele &

New 1974; New et al 1976). The sub-optimal development of embryos in untreated serum may result from a complement-dependent immune reaction between maternal antibodies and embryonic tissue because the natural barrier of Reichert's membrane and trophoblast were opened for the current experiment (Billington & Jenkinson 1975; Jensen et al 1975; Jensh et al 1977).

Supplementation of the medium with appropriate antibiotics, penicillin and streptomycin, is also important for the prevention of infections during experimental procedures.

6.1.4 Gas Phase and Rotating Bottle Culture Method

The oxygen content of the gas phase of the culture must be adjusted according to the stage of development of the embryos. For embryos of less than 10 somites, best results are obtained with 5-10% oxygen. This is equivalent to oxygen pressures found in the uterine vein and artery (Comline & Silver 1975; Yochim 1975). This is raised to 20-40% for embryos of 10-25 somites (New et al 1976). All the gas mixtures contain 5% CO_2 with the balance provided by nitrogen (New 1992). Thus, for our 48-hour culture, in the first 24 hours, 5%O_2; 5%CO_2; 90%N_2 were be used. In the next 8 hours, the gas phase was adjusted to 20%O_2; 5%CO_2; 75%N_2, followed by 40%O_2; 5%CO_2; 55N_2 in the remaining 16 hours of culture.

It is possible to maintain the correct gas phase by intermittent aeration of air-tight culture vessels which are placed in rotating incubators to ensure good equilibrium of the culture medium. However, the rotating-bottle culture unit (BTC Engineering, UK) was used in this study. This system has a central hollow rotating disk to which culture chambers / vessels are fixed to horizontally through silicone rubber bungs (Kochhar 1975;

Deuchar 1976). The disk revolves at a rate of 30-60 revolutions in each minute throughout culture period. Revolution is driven externally by an electric motor. Iinside this hollow rotating disk are ducts through which the oxygenating gas mixture circulates round the cavity of the disk and hence the culture chambers (New and Cockroft 1978). In order to ensure the smooth circulation of the oxygenating gas, the whole apparatus is a closed system, and any gas-loss is prevented by grease or with bungs. As for the gas flow rate, a few milliliters per minute is adequate. The entire rotator is made from aluminium and can be sterilized in a dry oven without dismantling. As for the glass rotating bottles, they can also be sterilized at high temperature.

The advantage of this rotating culture system are: 1) the continuous flow of filtered and humidified oxygenating gas ensures a stable O_2 and CO_2 composition and thus maintains a stable pH in the medium; 2) the continuous rotation of culture bottles ensures gas exchange between the culture medium and the gas phrase; 3) the gently swirling of the medium about the explants assists their respiration (New 1992); and 4) with the purpose-made automatic timer-switch, it ensures that the correct mixture of gas during different culture period is used.

6.2 Quantification of Oxidative Stress

6.2.1 8-isoprostaglandin F2α as a Marker

Direct measurement and quantification of free radical activity is difficult because of their extremely short half-lives. Free radical activity is directly related to the degree of tissue damage and therefore the level of product so generated. These products or metabolites are more stable then free radicals themselves and are therefore could be used as indicator of the degree of oxidative stress. However, most methods previously available to assess oxidant stress lacked sensitivity and specificity and are therefore unreliable (Halliwell & Grootveld 1987).

Isoprostanes, a family of eicosanoids, are a group of chemically stable compounds which are specific products from the reactive oxygen species-driven peroxidation of arachidonic acid residues in lipids, independent of the cyclooxygenase enzyme pathway (Morrow et al 1990). The notion that prostaglandin like compounds could be generated in vitro and in vivo non-enzymatically as products of autoxidation of fatty acids was actually first demonstrated over 20 years ago by autoxidation of plasma arachidonic acid in vitro during storage (Pryor et al 1976; Morrow et al 1990). Abstraction of hydrogens of arachidonic acid by free radicals leads to the formation of the three arachidonoyl radicals. Subsequent attack by O_2 results in formation of four peroxyl radical derivatives of arachidonic acid. These peroxyl radical derivatives then undergo endocyclization followed by further addition of O_2 to form PGG_2-like cyloendoperoxides. Reduction of these PGG_2-like compounds results in the formation of PGF_2-like compounds. Depending on the location of the peroxyl radical derivatives of arachidonic acid, four regioisomers are formed. Each of these regioisomers can be theoretically comprised of eight

racemic diastereomers. Since these compounds contain the F-type prostane ring and are isomeric to PGF2α, derived from the cyclooxygenase enzyme, they have been termed F2-isoprostane (Roberts & Morrow 1994).

At least one of the isoprostanes, 8-isoprostane (8-isoprostaglandin F2α), has been shown to have biological activity. There are growing evidence that 8-isoprostaglandin F2α are stable products of free radicals and therefore have been considered to be reliable markers of oxidative stress (Awad et al 1996; Roberts & Morrow 1994). Although some food does contain isoprostane, recent studies suggest that the amount present is insufficient to alter plasma levels even on the assumption that all the isoprostanes could be absorbed. I have therefore chosen 8-isoprostaglandin F2α assay as the indicator of oxidative stress for this study.

6.2.2 Assay for 8-isoprostaglandin F2α

6.2.2.1 Enzyme Immunoassay versus Gas Chromatography/ Mass Spectrometry

One of the ways to measure isoprostanes is a negative ionization Gas Chromatography/ Mass Spectrometry (GC/MS) assay. This method is highly sensitive with a lower limit of detection in the low picogram range and is highly accurate. Measurement of esterified levels of isoprostanes in tissues is accomplished by measurement of free compounds following alkaline hydrolysis of a lipid extract of tissue (Morrow & Roberts 1994). However, the mass spectrometric method of assay is highly labor intensive during the sample purification process. The instrument is expensive and it is costly to maintain. This method is therefore not

widely used in the scientific research. Recently, a number of enzyme immunoassays (EIA) have been developed and become commercially available, making the measurement of isoprostanes available to many more investigators (Proudfoot et al 1999; Wang et al 1995). I have therefore chosen the EIA method for the assay of 8-isoprostaglandin F2α.

EIA assay is based on the competition between 8-isoprostane and a 8-isoprostane-acetylcholinesterase conjugate (8-isoprostane tracer) for a limited amount of 8-isoprostane polyclonal antiserum. Because the concentration of the 8-isoprostane tracer is held constant while the concentration of 8-isoprostane varies, the amount of 8-isoprostane tracer that is able to bind to the 8-isoprostane polyclonal antiserum will be inversely proportional to the concentration of 8-isoprostane in the well. This antibody-8-isoprostane complex binds to a mouse anti-rabbit IgG monoclonal antibody that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's Reagent (which contains the substrate to acetylcholineseterase) is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs light strongly at wavelength of 412nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of 8-isoprostane tracer bound to the well, which is inversely proportional to the amount of free 8-isoprostane present in the well during the incubation.

Section III: Results

Chapter 7: Results

7.1 Justifications of Methods of Statistical Analysis

Our data, which had been estimated using Blom's method, was not normally distributed. Transformation of original data using logarithm, square root, exponential or square failed to yield a data set with normal distribution. Thus, the statistical analysis method used in this thesis to determine differences among groups was the nonparametric Kruskal-Wallis test, followed by the Least Significant Difference (LSD) test. The LSD test acts as a post-hoc test here to do pair wise comparisons between two groups. The trend analysis for these nonparametric data, was performed by using the nonparametric Spearman correlation which gives correlation coefficients as well as the correlation significance.

7.2 Effects of Hyperglycemia on Early Embryogenesis

7.2.1 Effects of Hyperglycemia on Morphological Development

There were totally 4 groups in this part of study. Based on studies performed by Norbert et al (Norbert et al 1986), a minimum sample size of 39 embryos in each group is required to detect a significant reduction in morphological scores under hyperglycemic condition. The final sample size for the control group, two-fold hyperglycemic group, four-fold hyperglycemic group and eight-fold hyperglycemic group were 48, 47, 41 and 41 respectively.

The findings of morphology scores of embryos are summarized in Table 1 and Table 2. Figure 1 to figure 4 are images of embryos after culture under different glycemic environments. Our data confirmed previous findings (Freinkel et al 1996; Reece et al 1998) that embryos cultured in higher glucose concentrations demonstrate significant more malformations compared to those cultured under normal conditions. This different applied to all morphological features assessed, including the number of somites, crown rump length and mean yolk sac diameter. Our data also confirmed that there was a dose dependent trend of teratogenic effects of hyperglycemia.

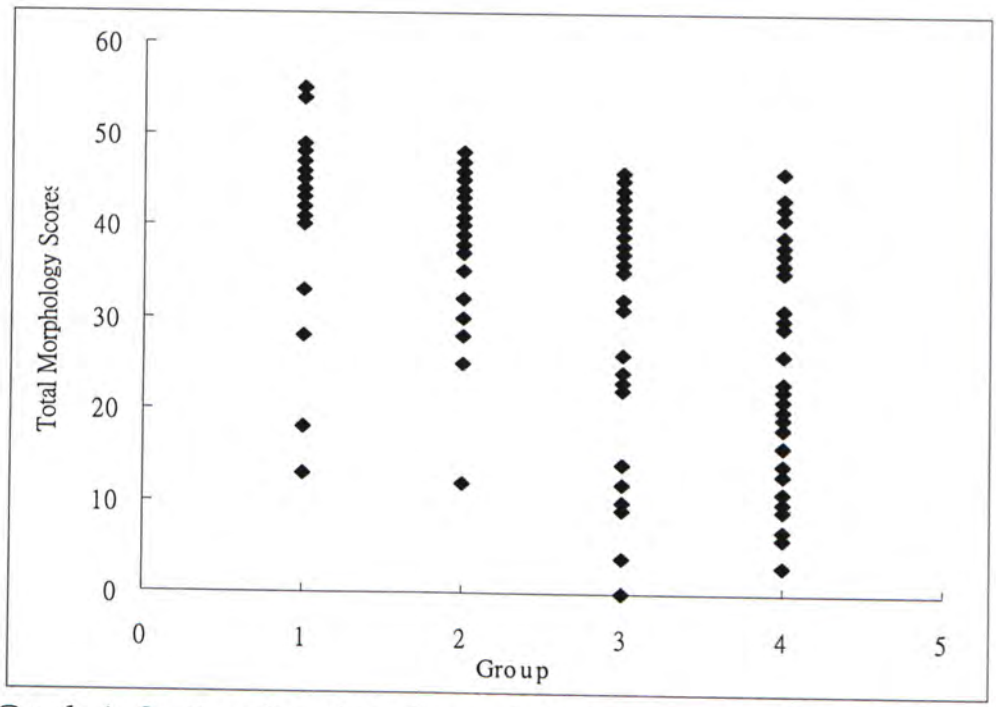
Table 1. Total morphology score, crown rump length, mean yolk sac diameter and number of somites of embryos cultured in various hyperglycemic conditions. Data are expressed in median (range) and * denotes $p<0.05$ in Least Significant Difference (LSD) test.

	Glucose Level				
	6.67 mmol/l (control) (n= 48)	13.30 mmol/l (n= 47)	26.70 mmol/l (n= 41)	53.40 mmol/l (n= 41)	Kruskal-Wallis test *p* value
Total Morphology Score	45 (13-55)	42 (12-48)*	38 (4-46)*	23 (4-46)*	<0.01
Crown Rump Length (mm)	3.5 (2.1-4.3)	3.5 (2.8-4.5)	3.3 (2.3-4.0)	3.2 (1.6-4.1)*	<0.01
Mean Yolk Sac Diameter (mm)	3.2 (2.1-4.0)	3.1 (2.4-3.9)	3.0 (1.9-4.0)	2.9 (1.6-4.0)*	<0.01
Number of Somites	24 (3-30)	22 (9-26)*	21 (4-30)*	15 (0-25)*	<0.01

Table 2. The composed morphology features as well as their scores to the total morphology scores of embryos cultured in various hyperglycemic levels.

Data are expressed in median (range) and * denotes $p<0.05$ in Least Significant Difference (LSD) test.

	Glucose Level				
	6.67 mmol/l (control) (n= 48)	13.30 mmol/l (n= 47)	26.70 mmol/l (n= 41)	53.40 mmol/l (n= 41)	Kruskal-Wallis test *p* value
Yolk Sac Circulation	3 (2-5)	3 (1-4)	2 (0-4)*	2 (1-4)*	<0.01
Allantois	2 (1-3)	2 (1-2)	2 (0-2)	1 (0-2)*	<0.01
Flexion	4 (0-5)	4 (0-5)	4 (0-5)	0 (0-4)*	<0.01
Heart	4 (2-5)	4 (2-4)	4 (1-4)	3 (1-4)*	<0.01
Caudal Neural Tube	5 (1-5)	5 (1-5)	5 (0-5)	3 (0-5)*	<0.01
Hind Brain	4 (1-5)	4 (1-4)	4 (1-4)	3 (0-4)*	<0.01
Mid Brain	4 (1-5)	4 (1-4)	4 (1-4)	3 (0-4)*	<0.01
Fore Brain	4 (1-5)	4 (1-4)	3 (1-4)*	2 (0-4)*	<0.01
Optic System	3 (1-3)	3 (0-3)	2 (0-3)*	1 (0-3)*	<0.01
Otic System	3 (0-4)	3 (0-3)	3 (0-3)*	1 (0-3)*	<0.01
Branchial Bars	2 (1-3)	2 (1-2)	2 (0-2)	1 (0-2)*	<0.01
Fore Limb	2 (0-3)	2 (0-2)	2 (0-2)	1 (0-2)*	<0.01
Hind Limb	2 (0-2)	1 (0-2)*	0 (0-2)*	0 (0-2)*	<0.01



Graph 1. Scatter plot of total morphology scores in different glucose levels. Embryos cultured in groups 1 to 4 with glucose concentrations of 6.67mM, 13.30mM, 26.70mM and 53.40mM, respectively.

Figure 1a.

Embryo after 48 hours of culture in standard culture medium with normoglycemic level (6.67 mmol/l D-glucose). E, EA, BB, H, and S represent eye, ear, branchial bar, heart, and somite respectively.

Figure 1b.

Embryo and its yolk sac after 48 hours of culture in standard culture medium with normoglycemic level (6.67 mmol/l D-glucose). YS and EC represent yolk sac and ectoplacental cone. Interval of scale bar = 1mm.

Figure 2a.

Embryo after 48 hours of culture under 2-fold hyperglycemic condition (13.30 mmol/l D-glucose). E, BB, H, FL, and EA represent eye, branchial bar, heart, forelimb, and ear respectively.

Figure 2b.

Embryo and its yolk sac after 48 hours of culture under 2-fold hyperglycemic condition (13.30 mmol/l D-glucose). YS and EC represent yolk sac and ectoplacental cone. Interval of scale bar = 1mm.

Figure 3.

Embryo after 48 hours of culture under 4-fold hyperglycemic condition (26.70 mmol/l D-glucose). E, BB, H, EA, and CNT represent eye, branchial bar, heart, ear and caudal neural tube respectively.

Figure 4a.

Embryo after 48 hours of culture under 8-fold hyperglycemic condition (53.40 mmol/l D-glucose). EA, H, BB, and E represent ear, heart, branchial bar, and eye respectively.

Figure 4b.

Embryo and its yolk sac after 48 hours of culture under 8-fold hyperglycemic condition (53.40 mmol/l D-glucose). EC and YS represent ectoplacental cone and yolk sac. Interval of scale bar = 1 mm.

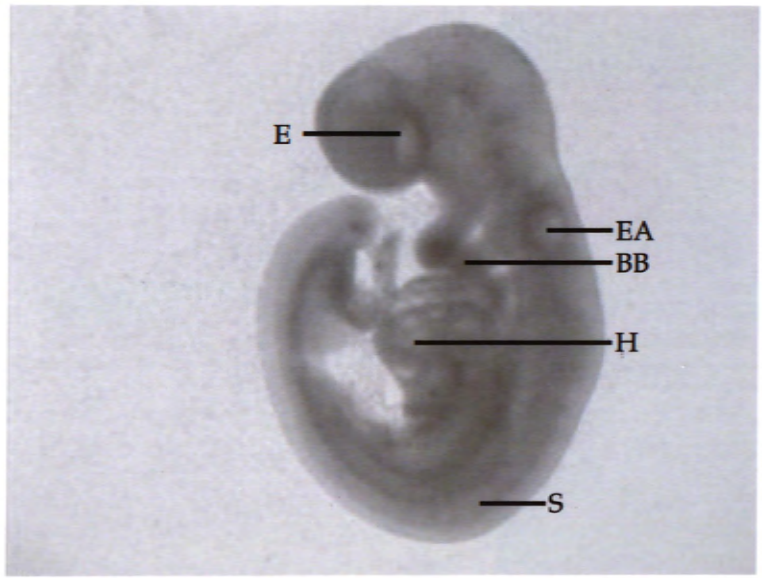


Figure 1a

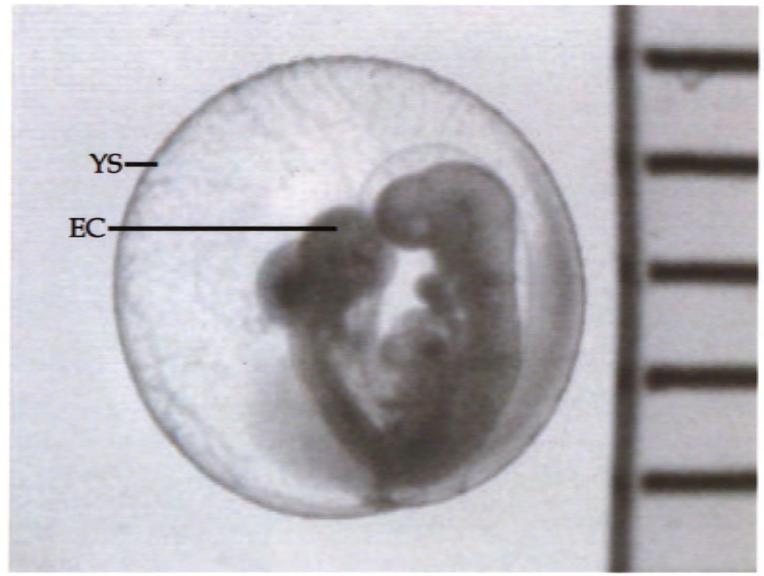


Figure 1b

Figure 2a

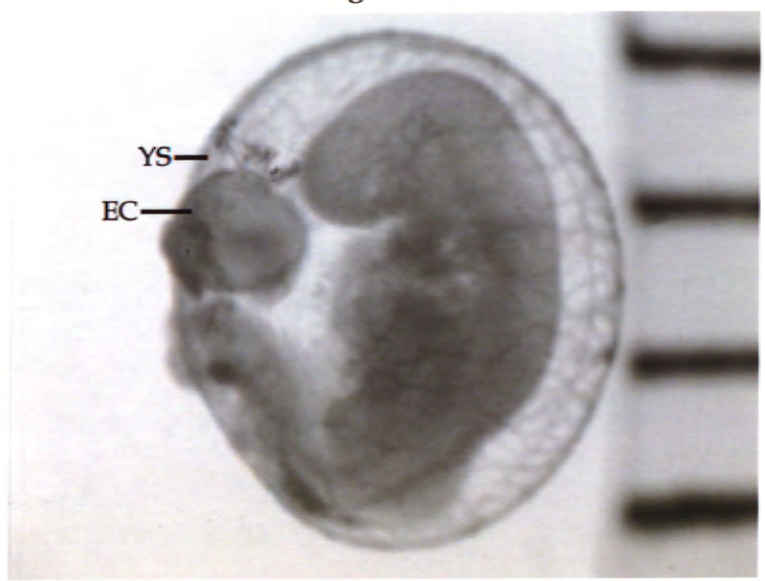


Figure 2b

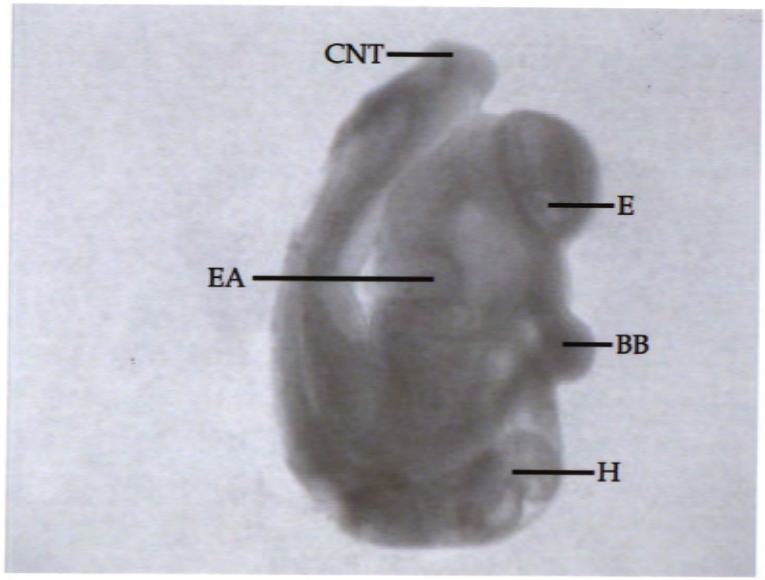


Figure 3

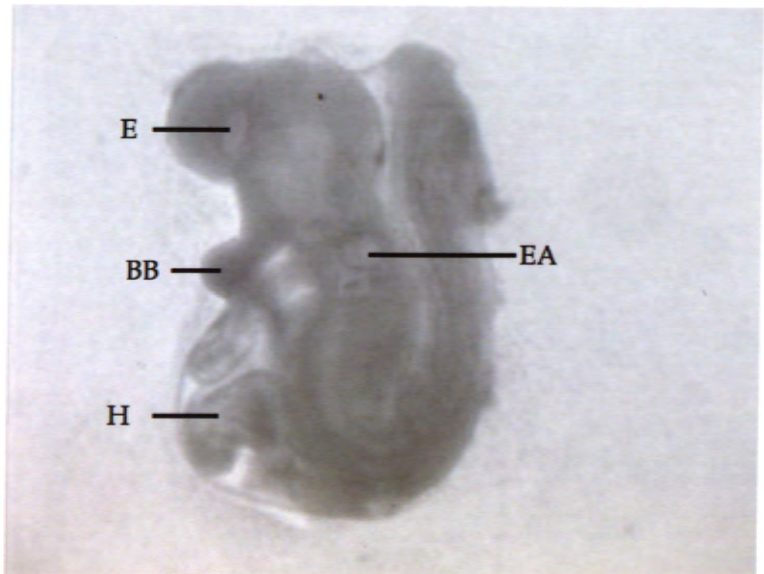


Figure 4a

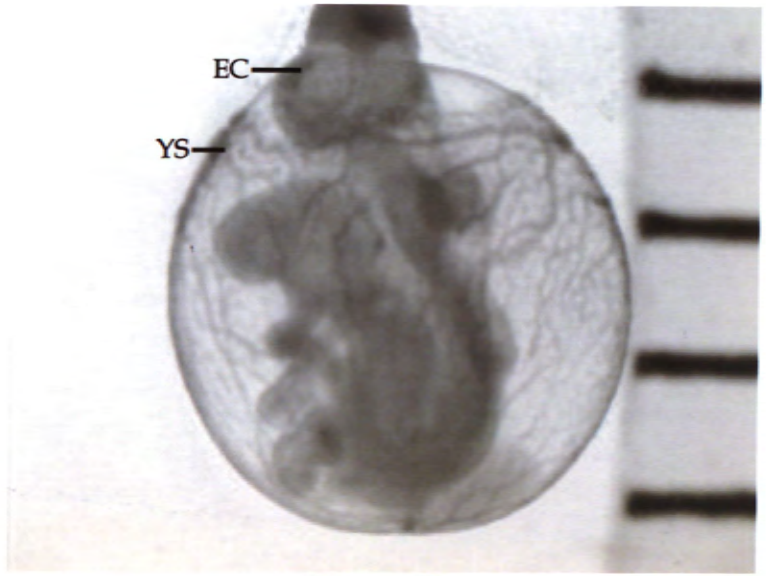


Figure 4b

7.2.2 Effects of Hyperglycemia on Production of 8-isoprostaglandin F2α

In embryos and yolk sacs

It was postulated that biochemical changes under different conditions, such as the changes of 8-isoprostaglandins F2α, should be more profound that morphological changes. Therefore, a smaller sample size may be adequate to detect such differences. Based on previous reported experiments (Wentzel et al 1999), 7-10 embryos from each experimental group would be adequate for the quantification of oxidative stress.

10 embryos were randomly selected from each of the experimental groups (control group, two fold hyperglycemic group, four fold hyperglycemic group and eight fold hyperglycemic group) and were subjected to 8-isoprostaglandins F2α extraction and assay. The unit expressed was the amount of isoprostane in picograms per unit length of embryo in millimeters (crown rump length). The 8-isoprostaglandins F2α concentrations of the corresponding yolk sacs from these 10 embryos were similarly measured, and the unit expressed was the amount of isoprostane in concentration of picograms per unit length of mean yolk sac diameter. Results for this part of the study are shown in Table 3.

Our data showed that both embryos and yolk sacs overproduce 8-isoprostaglandins F2α, per unit length, under increasing glucose level conditions. These increases were significant by Kruskal-Wallis test.

According to the nonparametric Spearman correlation test, there was a significant reverse correlation ($p=0.007$) between concentrations of 8-isoprostaglandin F2α produced in embryos and the total developmental morphology scores of the embryos, with a correlation coefficient

was -0.418. Similar relationship was also observed between the yolk sac 8-isoprostaglandins F2α concentration and the total morphology scores of embryos (Spearman correlation of -0.519, p=0.001).

In culture medium

We have collected all culture media immediately after 48-h in vitro culture and stored them at -78°C until assayed. Since every 2 to 4 embryos of the same group would be chambered in the same culture bottle during experiment, throughout the experiment, we had 18, 18, 16 and 17 bottles of culture media for control group, two-fold hyperglycemic group, four-fold hyperglycemic group, and eight-fold hyperglycemic group, respectively.

Unlike that with yolk sacs or embryos, no statistically significant difference in 8-isoprostaglandin F2α levels could be detected in the culture media between groups (Table 3). Based on these data, we conclude that culture media are not reflective of oxidative stress of embryos during culture. The range within every group was also unacceptably large. We thought that the most plausible reason was as follow. Rats were anesthetized before sera were collected. There was possibly a large difference in the degree of oxidative stress during the process of anesthesia, and as a result, a large difference in isoprostane levels between different rat sera samples. These inherent differences could have masked any potential difference contributed by different culturing environment between the experimental groups. Therefore, assay of culture medium was subsequently not performed for other parts of the study.

Table 3. Levels of 8-isoprostaglandins F2α produced in embryos, yolk sacs, and culture medium after culture in different hyperglycemic conditions. Data are expressed in median (range) and * represents $p<0.05$ when means of groups are compared by Least Significant Difference (LSD) test.

	Glucose Levels				
	6.67 mmol/l (control) (n=10)	13.30 mmol/l (n=10)	26.70 mmol/l (n=10)	53.40 mmol/l (n=10)	Kruskal-Wallis Test *p* Value
8-Isoprostaglandins F2α (pg/mm) in embryo	3.00 (2.51-3.94)	3.92 (0.49-7.33)	5.31 (2.79-9.28)*	6.66 (2.33-12.41)*	0.015
8-Isoprostaglandins F2α (pg/mm) in yolk sac	1.83 (1.43-5.60)	3.87 (1.60-6.76)	3.96 (1.76-6.37)	11.02 (4.63-12.10)*	<0.01
8-Isoprostandins F2α (pg/ml) in culture medium	53.97 (9.47-129.64)	63.69 (7.24-172.31)	55.43 (14.74-194.00)	67.46 (8.05-223.65)	0.526

7.2.3 Effects of Hyperglycemia on Total Protein Content

All samples of embryos and yolk sacs after culture were used. Some sample were lost during the experimental procedures. Therefore, the sample sizes for embryos were not the same as that for yolk sacs. The exact sample sizes for each group were shown in Table 4.

As shown in Table 4, there was no difference between different experimental groups in total protein content in either the embryos or the yolk sacs. However, there were direct and positive relationships between total embryonic protein contents and total morphology scores by Spearman correlation (p <0.01, r^2=0.319), and between total embryonic protein contents and embryonic crown rump length (p <0.01, r^2= 0.341). Similarly, there were significant and positive correlations between yolk sac protein contents and total morphology score (p = 0.042, r^2= 0.155), and between yolk sac protein content and mean yolk sac diameter (p <0.01, r^2= 0.397).

Table 4. Total protein content in embryos and yolk sacs after 48-h culture in different hyperglycemic conditions. Data are expressed in median (range).

	Glucose Level				
	6.67 mmol/l (control)	13.30 mmol/l	26.70 mmol/l	53.40 mmol/l	Kruskal-Wallis Test *p* Value
Total protein content (μg) in embryo	489.22 (199.89-1269.13) (n=48)	412.88 (159.38-876.50) (n=47)	457.16 (33.38-1023.25) (n=41)	103.13 (112.74-823.88) (n=43)	0.179
Total protein content (μg) in yolk sac	446.70 (243.30-815.57) (n=48)	416.10 (265.50-763.43) (n=46)	487.21 (244.86-812.86) (n=42)	416.71 (21.30-727.27) (n=43)	0.084

7.3 Effects of Caffeine on Early Embryogenesis

7.3.1 Effects of Caffeine on Morphological Development

There were totally 6 groups in this part of study: the experimental control group, the 10μg/ml caffeine group, the 20μg/ml caffeine group, the 30μg/ml caffeine group, the 60μg/ml caffeine group, and the 90μg/ml caffeine group. Similar to the experiment with hyperglycemia, we aimed for about 40 embryos per experimental group. Since there were only 30 embryos available for experiment each time, it was decided to split this part of study into two stages so that the number of embryos in each group during any experiment would not be too few - the first trial distributed embryos to either control group, 30μg/ml caffeine group, 60μg/ml caffeine group and 90μg/ml caffeine; the second trial was to distribute embryos to either control group, 10μg/ml caffeine group and 20μg/ml caffeine group. The first part aimed at testing the effect of high caffeine concentration, while the second part aimed at lower caffeine concentration. Therefore, the number of embryos in the control group was approximately twice that of the other experimental groups. Ultimately, we had 92, 42, 40, 46, 48 and 34 embryos in the control group, the 10μg/ml caffeine group, the 20μg/ml caffeine group, the 30μg/ml caffeine group, the 60μg/ml caffeine group and the 90μg/ml caffeine group respectively. Since the results of the control groups between the two trials were similar, data were pooled together for data analysis so that between group comparisons would be easier to appreciate.

Morphology scores were summarized in Table 5 and Table 6. Figures 5 to 9 are images of embryos subjected to different doses of caffeine. Our data showed that there were significant between group differences in all morphological features assessed, including size of the yolk sac and

embryo, total morphological score and all individual morphological scores. Specifically, virtually all morphological features assessed were significantly smaller or lower when exposed to 60μg/ml of caffeine or above. On the other hand, there was virtually no difference between the control group and those exposure to caffeine up to 20μg/ml. It therefore appeared that threshold teratogenic dose of caffeine was around 60μg/ml. However, size of yolk sac and number of somites seemed to be more vulnerable because they showed significant reduction in size and number respectively at a caffeine dose of 30μg/ml.

For the second part of the present study, we did not assay or quantify 8-isoprostaglandin F2α levels. We consider that such assay was unnecessary because if caffeine does act as antioxidant, its 8-isoprostaglandin F2α levels would be lower than that of baseline levels (control cases). Technically, it would be difficult to detect isoprostane levels lower than 3.0 pg/ml (the baseline levels in control group based on the hyperglycmia experiment). Furthermore, the result of this assay would not provide extra information for the hypothesis to be test i.e. caffeine as antioxidant in preventing hyperglycemia-induced teratogenecity.

Table 5. Total morphology score, crown rump length, mean yolk sac diameter and number of somites of embryos cultured in various caffeine levels.

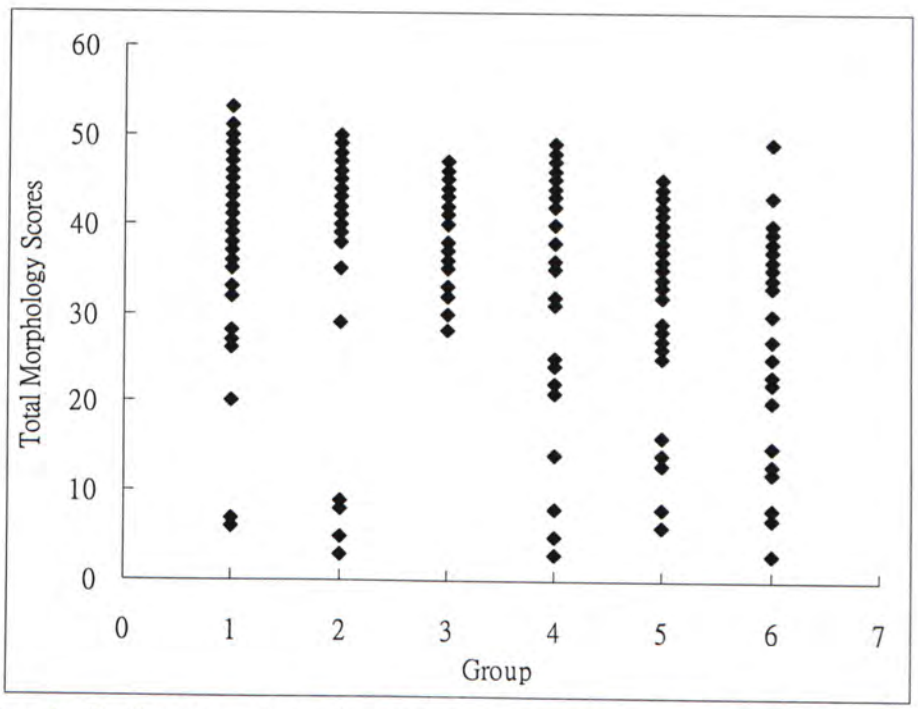
Data are expressed in median (range) and * denotes $p<0.05$ in Least Significant Difference (LSD) test.

	Caffeine Level						
	0μg/ml (control) (n= 89)	10μg/ml (n= 41)	20μg/ml (n= 41)	30μg/ml (n= 44)	60μg/ml (n= 47)	90μg/ml (n= 33)	Kruskal-Wallis Test *p* value
Total Morphology Scores	46 (6-53)	44 (5-50)	43 (28-47)	43.5 (5-49)	36 (6-45)*	30 (7-49)*	<0.01
Crown Rump Length (mm)	3.4 (1.0-4.3)	3.4 (1.3-4.0)	3.2 (2.5-4.0)	3.1 (1.3-3.9)	3.1 (1.2-4.3)*	3.0 (1.6-3.6)*	<0.01
Mean Yolk Sac Diameter (mm)	4.0 (1.5-4.2)	4.4 (1.5-5.2)	4.0 (3.1-5.2)	3.7 (1.9-4.5)*	3.6 (2.5-4.5)*	3.7 (2.2-4.5)*	<0.01
Number of Somites	22 (4-28)	23 (0-26)	21 (13-24)	22 (3-26)*	21 (4-25)*	16 (0-23)*	<0.01

Table 6. The composed morphology features as well as their scores to the total morphology scores of embryos cultured in various caffeine levels.

Data are expressed in median (range) and * denotes $p<0.05$ in Least Significant Difference (LSD) test.

	Caffeine Level						
	0 µg/ml (control) (n= 89)	10 µg/ml (n= 41)	20 µg/ml (n= 41)	30 µg/ml (n= 44)	60 µg/ml (n= 47)	90 µg/ml (n= 33)	Kruskal-Wallis Test *p* value
Yolk Sac Circulation	4 (1-5)	3 (1-4)	3 (1-4)	3 (1-4)	2 (1-4)*	2 (1-4)*	<0.01
Allantois	2 (0-2)	2 (0-2)	2 (1-2)	2 (0-2)	1 (0-2)*	1 (0-2)*	<0.01
Flexion	4 (0-5)	4 (0-4)	4 (0-5)	4 (0-5)	4 (0-4)*	4 (0-4)	<0.01
Heart	4 (0-5)	4 (0-5)	4 (1-5)	4 (0-5)	3 (1-4)*	2 (1-4)*	<0.01
Caudal Neural Tube	5 (1-5)	5 (1-5)	5 (2-5)	5 (0-5)	4 (1-5)*	3 (1-5)*	<0.01
Hind Brain	4 (1-4)	4 (1-4)	4 (1-4)	4 (1-5)	3 (1-4)*	3 (1-4)*	<0.01
Mid Brain	4 (1-4)	4 (1-4)	4 (3-4)	4 (1-5)	3 (1-4)*	3 (1-4)*	<0.01
Fore Brain	4 (1-4)	4 (1-4)	4 (3-4)	4 (1-5)	3 (1-4)*	3 (1-4)*	<0.01
Optic System	3 (0-4)	3 (0-4)	3 (1-4)	3 (0-3)	2 (0-3)*	2 (0-3)*	<0.01
Otic System	3 (0-4)	3 (0-4)	3 (2-4)	3 (0-4)	2 (0-3)*	2 (0-3)*	<0.01
Branchial Bars	2 (0-3)	2 (0-3)	2 (2)	2 (1-2)	2 (0-2)	2 (1-3)	<0.01
Fore Limb	2 (0-3)	2 (0-3)	2 (0-3)	0 (0-3)	2 (0-2)	1 (0-2)*	<0.01
Hind Limb	1 (0-2)	1 (0-2)	1 (0-2)	1 (0-2)*	0 (0-2)*	0 (0-2)*	<0.01



Graph 2. Scatter plot of total morphology scores in different caffeine concentrations. Where group 1 is control group. Embryos in groups 2 to 6 subjected to caffeine concentrations of 10μg/ml, 20μg/ml, 30μg/ml, 60μg/ml and 90μg/ml, respectively

Figure 5.

Embryo after 48 hours of culture in standard culture medium. EA, H, S, HL, BB, E, and FB represent ear, heart, somite, hindlimb, branchial bar, eye, and fore brain respectively. Interval of scale bar = 1mm.

Figure 6a.

Embryo after 48 hours of culture in 10 μg/ml caffeine. E, BB, EA, H, and HL represent eye, branchial bar, ear, heart, and hindlimb respectively.

Figure 6b.

Embryo and its yolk sac after 48 hours of culture in 10 μg/ml caffeine. EC, YS, and A represent ectoplacental cone, yolk sac, and allantois respectively. Interval of scale bar = 1mm.

Figure 7a.

Embryo after 48 hours of culture in 20 μg/ml caffeine. EA, H, FL, S, BB, and E represent ear, heart, forelimb, somite, branchial bar, and eye respectively.

Figure 7b.

Embryo and its yolk sac after 48 hours of culture in 20 μg/ml caffeine. YS and EC represent yolk sac and ectoplacental cone. Interval of scale bar = 1mm.

Figure 8a.

Embryo after 48 hours of culture in 30 μg/ml caffeine. E, FB, CTN, BB, H, S, and EA represent eye, forebrain, caudal neural tube, branchial bar, heart, somite, and ear respectively.

Figure 8b.

Embryo and its yolk sac after 48 hours of culture in 30 μg/ml caffeine. YS and EC represent yolk sac and ectoplacental cone respectively. Interval of scale bar = 1mm.

Figure 9a.

Embryo after 48 hours of culture in 60 μg/ml caffeine. HB, EA, S, H, BB, and E represent hindbrain, ear, somite, heart, branchial bar, and eye respectively.

Figure 9b.

Embryo and its yolk sac after 48 hours of culture in 60 μg/ml caffeine. EC, YS, and A represent ectoplacental cone, yolk sac, and allanotis respectively. Interval of scale bar = 1mm.

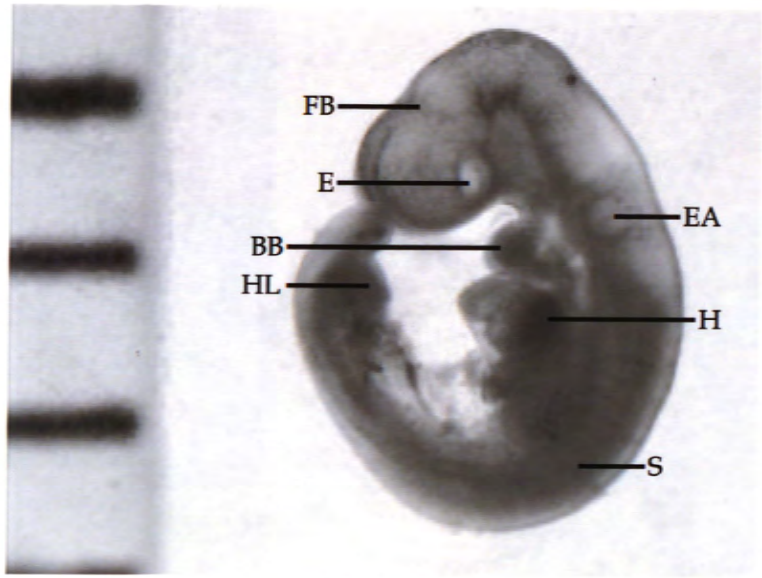


Figure 5

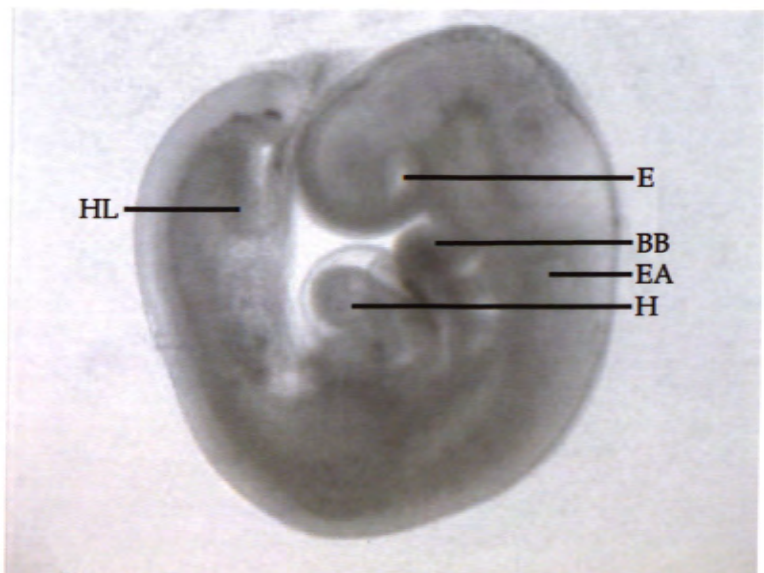


Figure 6a

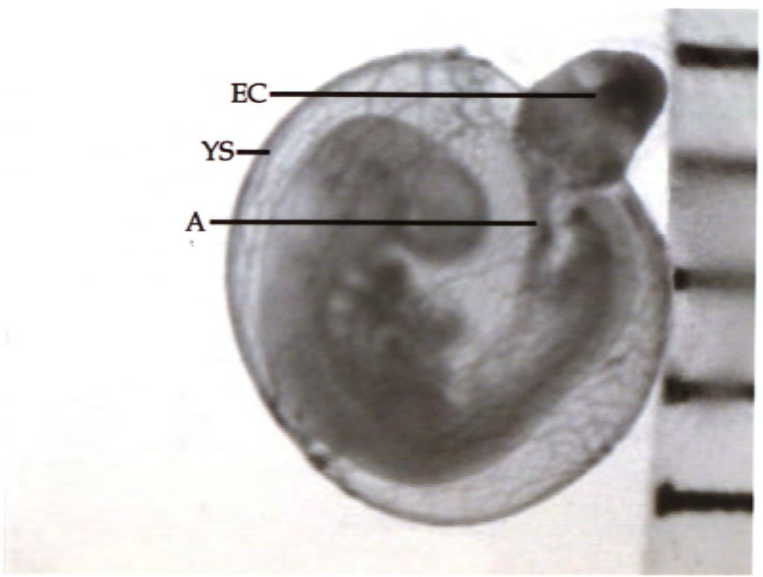


Figure 6b

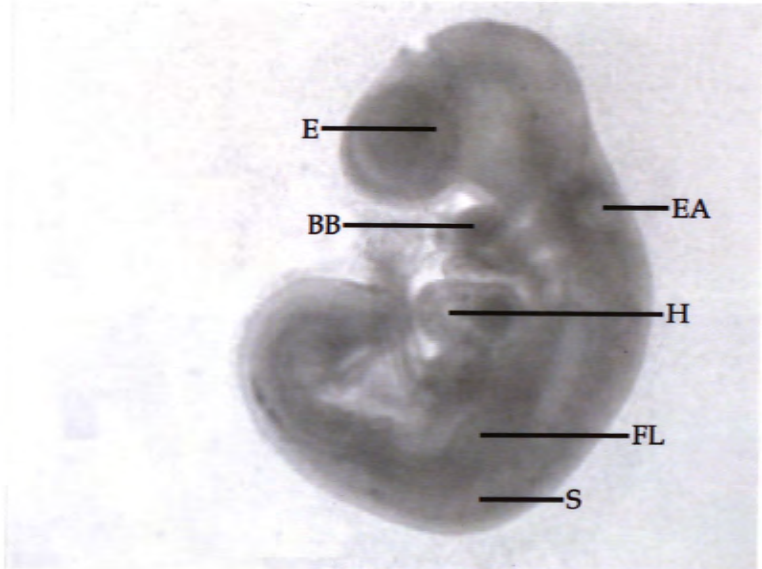


Figure 7a

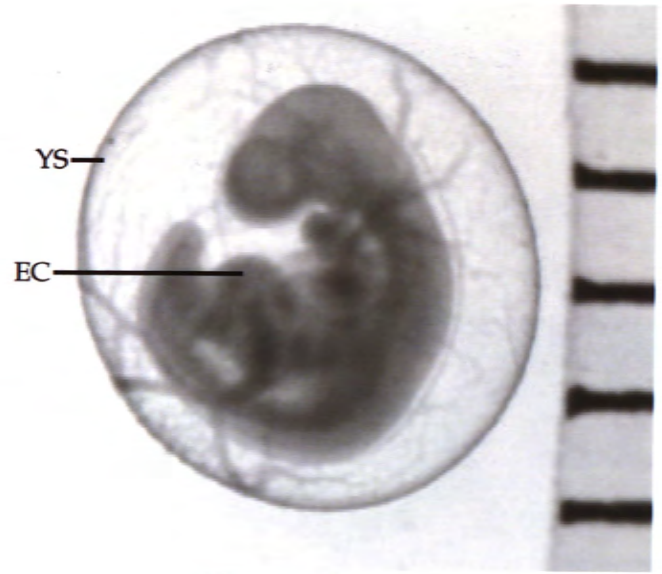


Figure 7b

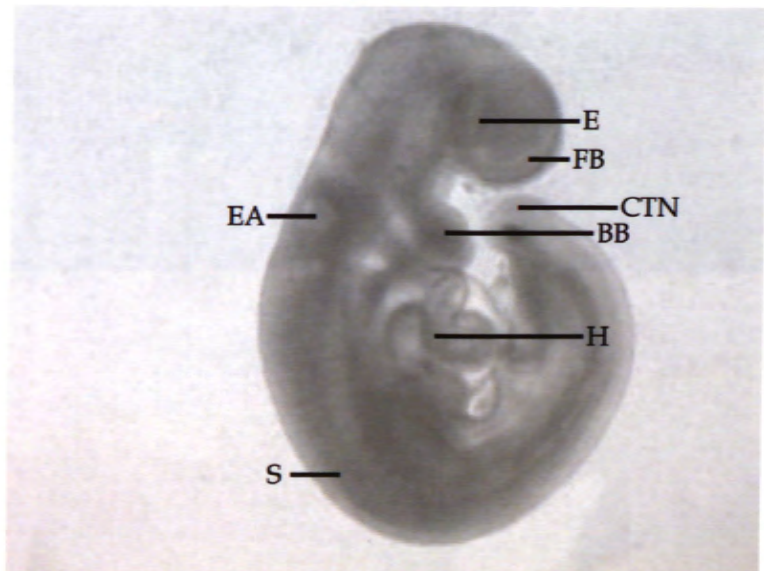


Figure 8a

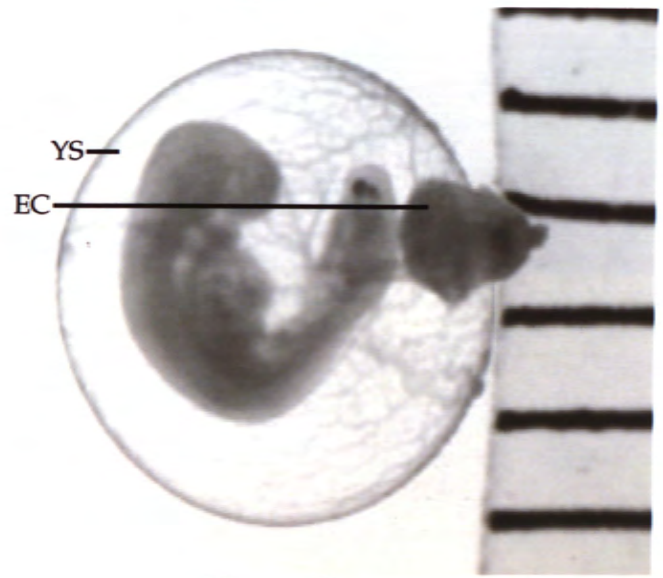


Figure 8b

Figure 9a

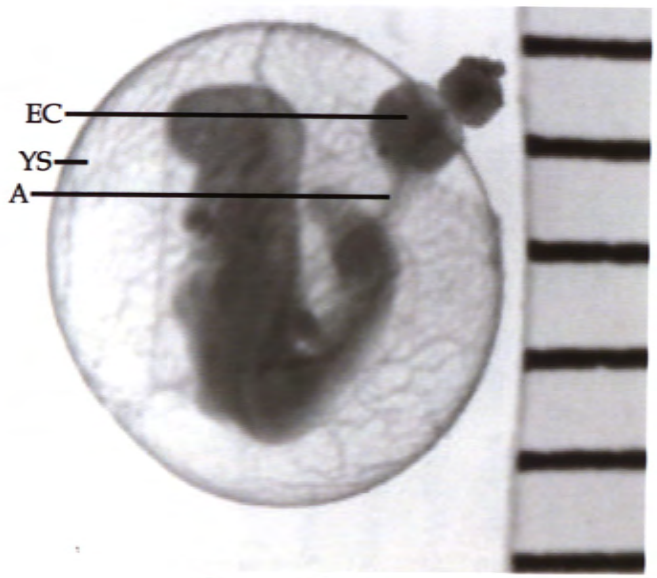


Figure 9b

7.3.2 Effects of Caffeine on Total Protein Content

All the samples harvested from culture were subjected to protein assay and the results are shown in Table 7. There was no between group difference in total protein content in embryos (Kruskal-Wallis Test, p=0.13). Although there was a significant between group difference in total yolk sac protein content, none of the caffeine group was found to be significantly different from the control group by the Least Significant Difference test.

However, there were significant and positive relationships between total embryonic protein content and total morphology score (p<0.01, r^2=0.446), and between total yolk sac protein content and total morphology score (p<0.01, r^2= 451) by Spearman correlation tests.

Table 7. Total protein content in embryos and yolk sacs after 48-h culture in different caffeine levels. Data are expressed in median (range).

	Caffeine Level						
	0μg/ml (control) (n= 92)	10μg/ml (n= 42)	20μg/ml (n= 40)	30μg/ml (n= 46)	60μg/ml (n= 48)	90μg/ml (n= 34)	Kruskal-Wallis Test *p* value
Total Protein Content (μg) in embryo	383.56 (84.89-599.65)	378.13 (153.79-665.47)	367.47 (211.05-559.68)	419.14 (159.47-679.70)	383.95 (176.10-630.50)	357.14 (124.20-582.70)	0.130
Total Protein Content (μg) in yolk sac	380.00 (121.80-703.79)	392.22 (146.78-690.53)	370.00 (145.79-663.89)	364.22 (162.20-615.30)	338.11 (102.00-524.11)	314.78 (130.52-533.79)	0.001

7.4 Combined Effects of Hyperglycemia and Caffeine on Early Embryogenesis

7.4.1 Combined Effects of Hyperglycemia and Caffeine on Morphological Development

As in the second part of the present study, we had split the samples into two parts. The first part included the control group, the four fold hyperglycemic group and the four fold hyperglycemia plus 1.0 μg/ml or 2.5 μg/ml caffeine groups. The second part included the control group, the four fold hyperglycemic group, the four fold hyperglycemia plus 5.0 μg/ml or 10.0 μg/ml caffeine groups. Again, we have pooled data from both parts for statistical analysis. Thus, the sample sizes of the control group and the four fold hyperglycemic group were approximately twice as many as other experimental groups. The sample size for the control group, the four fold hyperglycemic group, the hyperglycemia plus 1.0 μg/ml, 2.5 μg/ml, 5.0 μg/ml and 10.0 μg/ml caffeine groups were 39, 38, 16, 16, 24 and 21 respectively.

Morphology scores were summarized in Tables 8 and 9. Figures 10 to 15 are images of embryos subjected to different combinations of hyperglycemia and caffeine concentrations. All morphological features were smaller or lower in the hyperglycemic group compared with the control group. On the other hand, no difference in all morphological features was observed in any of the caffeine supplemented groups compared with the control, suggesting that hyperglycemia induced embryonic abnormality was preventable by caffeine supplementation.

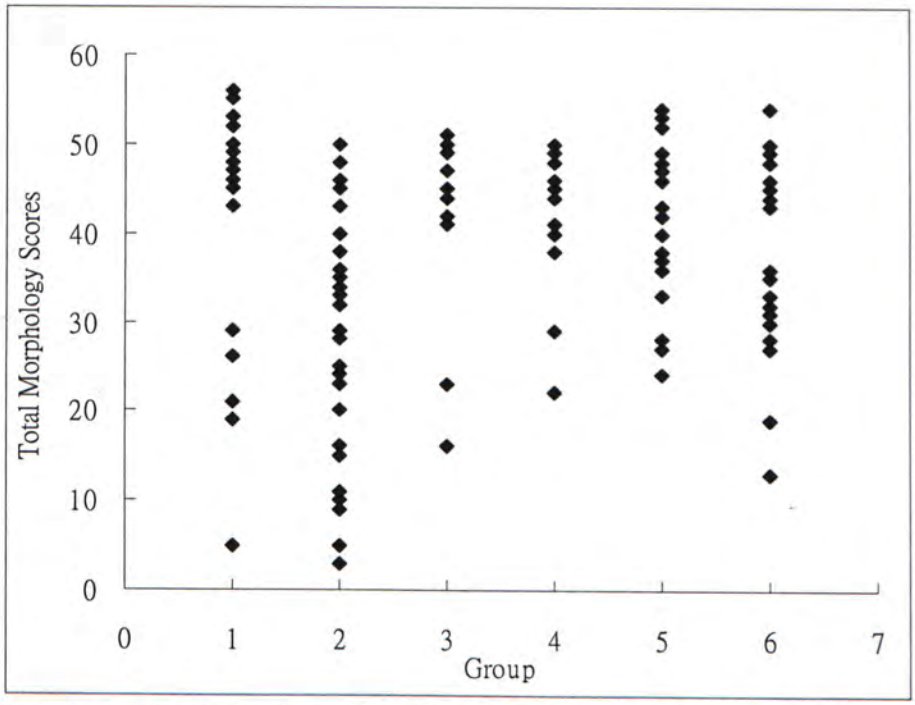
Table 8. Total morphology score, crown rump length, mean yolk sac diameter and number of somites of embryos cultured in various combined glucose and caffeine levels. Data are expressed in median (range) and * denotes $p<0.05$ in Least Significant Difference (LSD) test.

	Combined Glucose and Caffeine Level						
	Control (n= 39)	26.70mmol/l glucose (n=38)	26.70mmol/l glucose + 1.0 μg/ml caffeine (n= 16)	26.70mmol/l glucose + 2.5 μg/ml caffeine (n= 16)	26.70mmol/l glucose + 5.0 μg/ml caffeine (n= 24)	26.70mmol/l glucose + 10.0 μg/ml caffeine (n= 21)	Kruskal-Wallis Test *p* value
Total Morphology Scores	48 (5-56)	32.5 (5-50)*	45 (16-51)	44.5 (22-50)	43 (24-54)	44 (19-54)	<0.01
Crown Rump Length (mm)	4.0 (1.5-5.4)	3.5 (2.3-5.1)*	4.6 (3.0-5.5)	4.6 (3.5-5.2)	3.6 (2.3-4.5)	3.6 (3.0-4.1)	<0.01
Mean Yolk Sac Diameter (mm)	3.5 (1.0-4.2)	3.0 (1.2-7.0)*	3.5 (2.8-4.2)	3.6 (3.0-4.1)	3.1 (2.2-4.0)	3.2 (2.2-3.9)	<0.01
Number of Somites	23 (0-26)	18 (2-25)*	23 (14-24)	22 (17-24)	21 (11-25)	18 (11-25)	<0.01

Table 9. The composed morphology features as well as their scores to the total morphology scores of embryos cultured in combined glucose and caffeine levels.

Data are expressed in median (range) and * denotes $p<0.05$ in Least Significant Difference (LSD) test.

	Combined Glucose and Caffeine Level						
	Control (n= 39)	26.70 mM glucose (n=38)	26.70 mM glucose + 1.0 µg/ml caffeine (n= 16)	26.70 mM glucose + 2.5 µg/ml caffeine (n= 16)	26.70 mM glucose + 5.0 µg/ml caffeine (n= 24)	26.70 mM glucose + 10.0 µg/ml caffeine (n= 21)	Kruskal-Wallis Test *p* value
Yolk Sac Circulation	4 (1-5)	3 (1-4)*	4 (1-5)	4 (3-5)	3 (2-4)	4 (2-4)	<0.01
Allantois	2 (0-2)	2 (0-2)	2 (1-2)	2 (2-2)	2 (2-2)	2 (2-3)	<0.01
Flexion	4 (0-5)	2 (0-4)*	4 (0-5)	4 (0-5)	4 (0-5)	4 (0-4)	<0.01
Heart	4 (0-5)	2 (0-4)*	4 (1-5)	4 (1-4)	4 (1-5)	4 (1-5)	<0.01
Caudal Neural Tube	5 (1-5)	3 (0-5)*	4 (1-5)	4 (3-5)	4 (2-5)	4 (1-5)	<0.01
Hind Brain	4 (1-5)	3 (1-4)*	4 (1-4)	4 (2-4)	4 (2-5)	3 (2-5)	<0.01
Mid Brain	4 (1-5)	3 (1-4)*	4 (1-4)	4 (1-4)	4 (2-5)	3 (2-5)	<0.01
Fore Brain	4 (1-5)	3 (1-5)*	4 (2-5)	3 (1-4)	3 (2-5)	3 (2-5)	<0.01
Optic System	4 (0-4)	2 (0-4)*	4 (1-4)	4 (2-4)	4 (1-4)	4 (1-4)	<0.01
Otic System	3 (0-4)	2 (0-3)*	3 (0-4)	3 (1-3)	3 (1-4)	3 (1-3)	<0.01
Branchial Bars	2 (0-3)	2 (0-2)	2 (1-2)	2 (0-2)	2 (1-3)	2 (1-2)	<0.01
Fore Limb	3 (0-3)	1 (0-3)*	2 (1-3)	2 (1-3)	2 (1-3)	2 (1-3)	<0.01
Hind Limb	2 (0-2)	1 (0-2)*	2 (0-2)	2 (0-2)	1 (0-2)	1 (0-2)	<0.01



Graph 3. Scatter plot of total morphology scores in different hyperglycemic plus caffeine conditions. Where Group 1 is experimental control; Embryos in groups 2 to 6 with 26.70mM glucose plus caffeine concentrations of 1.0μg/ml, 2.5μg/ml, 5.0μg/ml and 10.0μg/ml, respectively.

Figure 10a.

Embryo after 48 hours of culture in standard culture medium. EA, H, S, BB, and E represent ear, heart, somite, branchial bar, and eye respectively. Interval of scale bar = 1mm.

Figure 10b.

Embryo and its yolk sac after 48 hours of culture in standard culture medium. EC, YS, A, BB, and H represent ectoplacental cone, yolk sac, allantois, branchial bar, and heart respectively. Interval of scale bar = 1mm.

Figure 11a.

Embryo after 48 hours of culture in combinations of 4-fold hyperglycemia and 1.0 μg/ml caffeine. HB, BB, S, FL, H, E, FB, CNT, and MB represent hindbrain, branchial bar, somite, forelimb, heart, eye, forebrain, caudal neural tube, and midbrain respectively. Interval of scale bar = 1mm.

Figure 11b.

Embryo and its yolk sac after 48 hours of culture in combinations of 4-fold hyperglycemia and 1.0 μg/ml caffeine. YS and EC represent yolk sac and ectoplacental cone. Interval of scale bar = 1mm.

Figure 12a.

Embryo after 48 hours of culture in combinations of 4-fold hyperglycemia and 2.5 μg/ml caffeine. FB, E, BB, HL, FL, S, H, and EA represent forebrain, ear, branchial bar, hindlimb, forelimb, somite, heart, and ear respectively. Interval of scale bar = 1mm.

Figure 12b.

Embryo and its yolk sac after 48 hours of culture in combinations of 4-fold hyperglycemia and 2.5 μg/ml caffeine. YS represent yolk sac. Interval of scale bar = 1mm.

Figure 13a.

Embryo after 48 hours of culture in combinations of 4-fold hyperglycemia and 5.0 μg/ml caffeine. E, BB, S, FL, H, and EA represent eye, branchial bar, somite, forelimb, heart, and ear respectively. Interval of scale bar = 1mm.

Figure 13b.

Embryo and its yolk sac after 48 hours of culture in combinations of 4-fold hyperglycemia and 5.0 μg/ml caffeine. YS, EC and A represent yolk sac, ectoplacental cone, and allantois. Interval of scale bar = 1mm.

Figure 14a.

Embryo after 48 hours of culture in combinations of 4-fold hyperglycemia and 10.0 μg/ml caffeine. EA, S, H, BB, E, and FB represent ear, somite, heart, branchial bar, eye, and forebrain respectively. Interval of scale bar = 1mm.

Figure 14b.

Embryo and its yolk sac after 48 hours of culture in combinations of 4-fold hyperglycemia and 10.0 μg/ml caffeine. YS represent yolk sac. Interval of scale bar = 1mm.

Figure 15.

Embryo after 48 hours of culture in 4-fold hyperglycemic conditions. CNT, E, BB, H, and EA represent caudal neural tube, eye, branchial bar, heart,

and ear respectively.

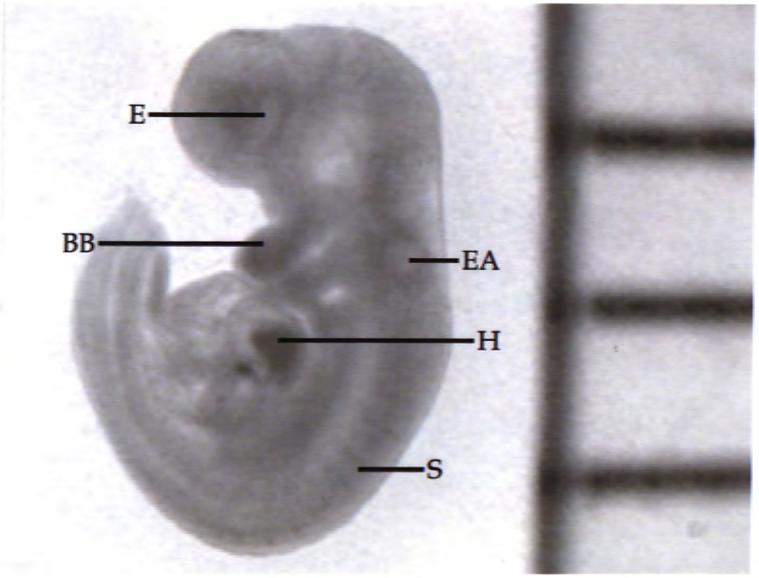


Figure 10a

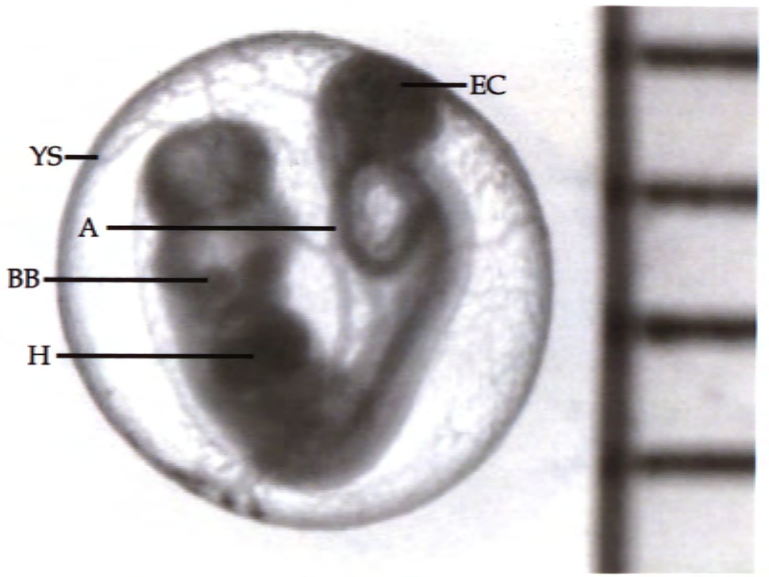


Figure 10b

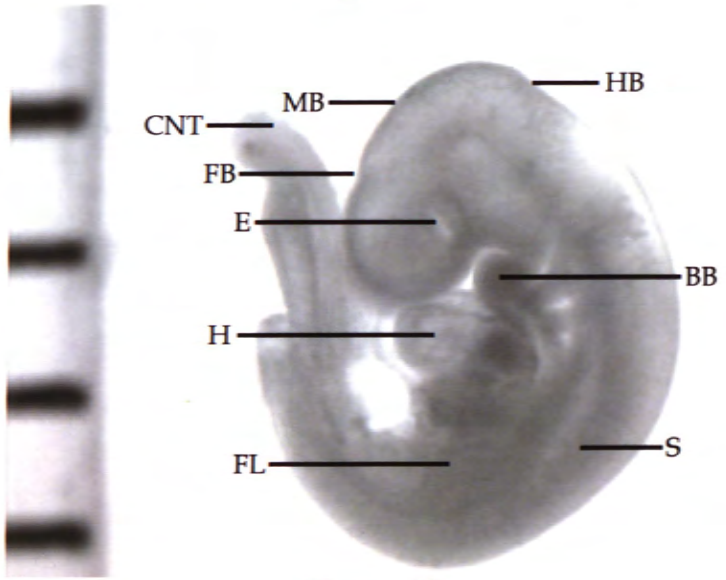


Figure 11a

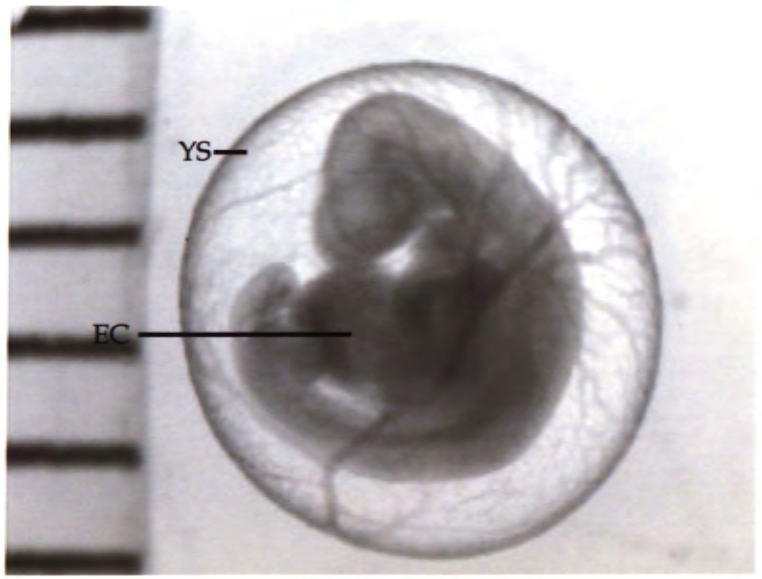


Figure 11b

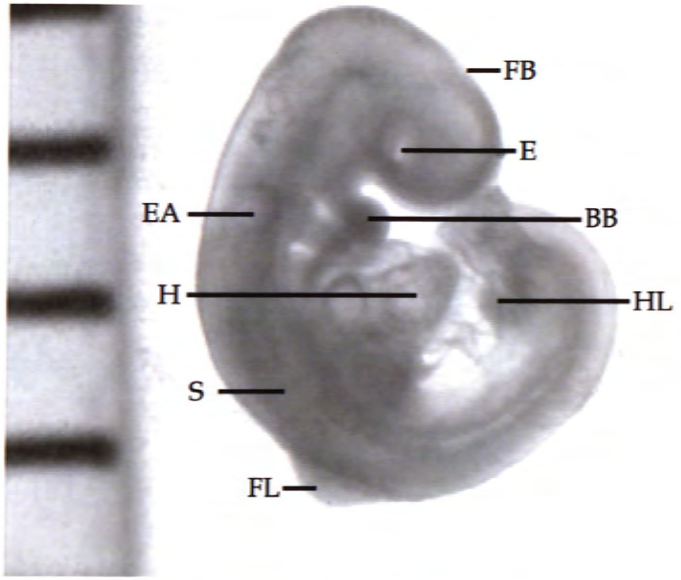


Figure 12a

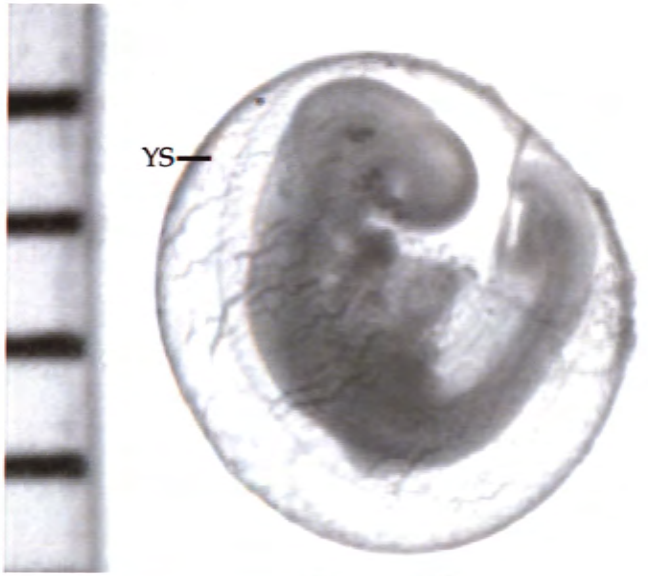


Figure 12b

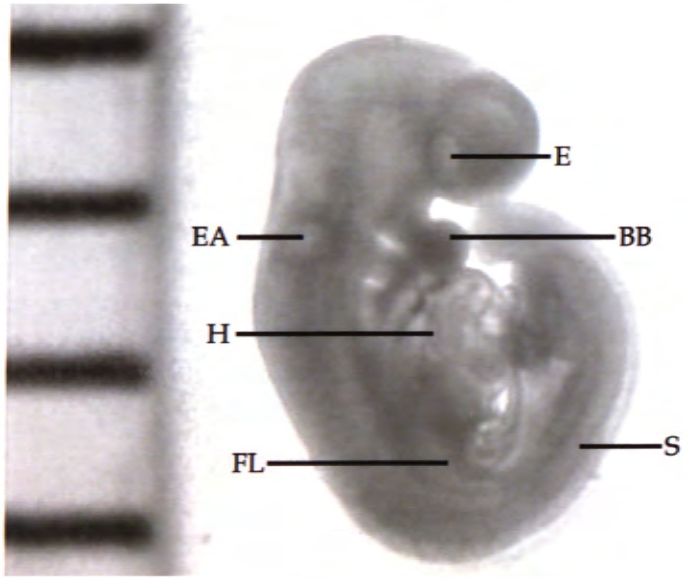


Figure 13a

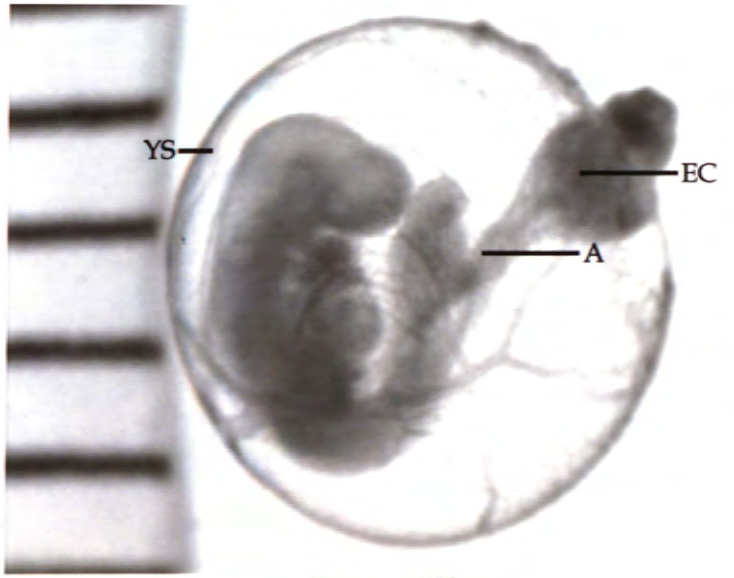


Figure 13b

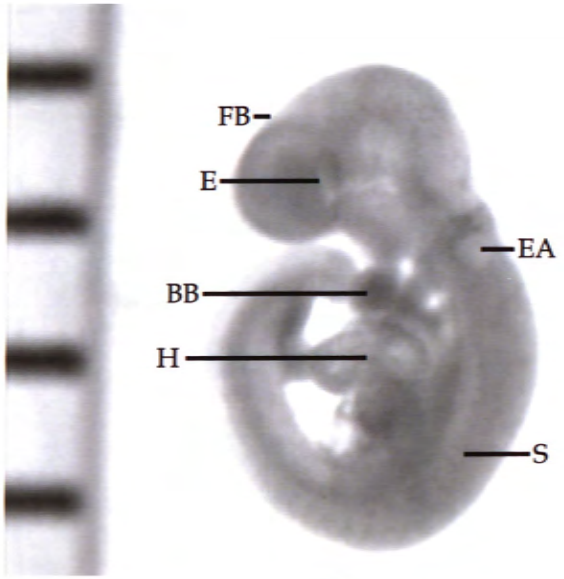


Figure 14a

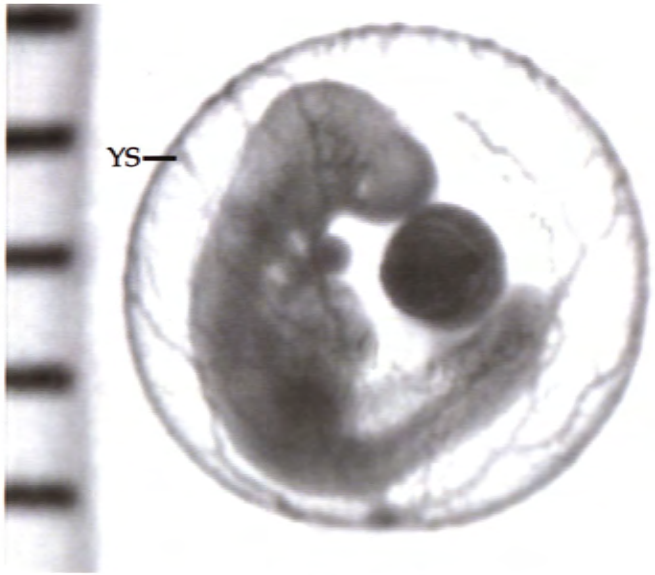


Figure 14b

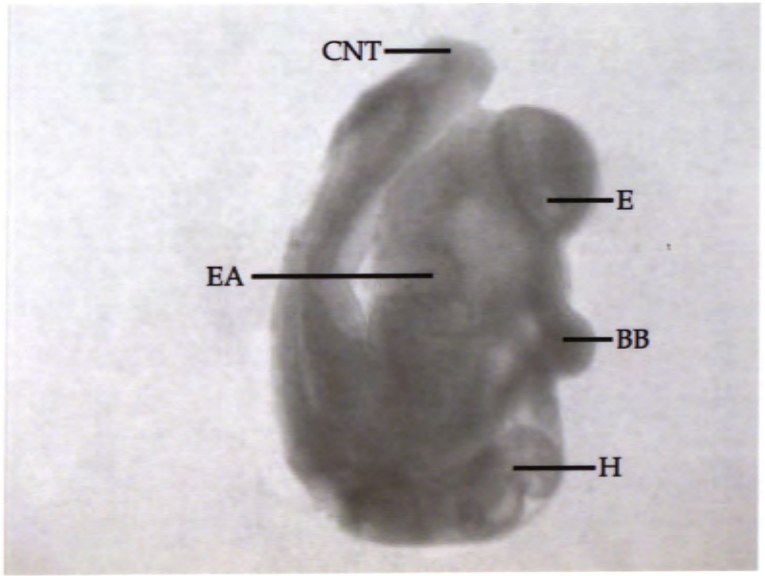


Figure 15

7.4.2 Combined Effects of Hyperglycemia and Caffeine on Production of 8-isoprostaglandin F2α

Results were summarized in Table 10. Compared with control, the concentrations of 8-isoprostaglandin F2α were significantly higher only in embryos and yolk sacs cultured in four-fold hyperglycemic conditions. This increased was prevented and reverted by the supplementation of caffeine. In fact, compared with the control, the supplementation with 5.0 μg/ml and 10.0μg/ml of caffeine caused a significant reduction in 8-isoprostaglandin F2α level in yolk sacs even in the presence of hyperglycemia.

In embryos, significant and negative correlations existed between levels of embryonic 8-isoprostaglandin F2α and total morphology scores ($p=0.001$, $r^2=-0.341$) and between levels of embryonic 8-isoprostaglandin F2α and crown rump length ($p=0.01$, $r^2=-0.268$). In the yolk sac, a significant negative correlation existed between levels of yolk sac 8-isoprostaglandin F2α and yolk sac diameter ($p=0.01$, $r^2=-0.480$).

7.4.3 Combined Effects of Hyperglycemia and Caffeine on Total Protein Content

Table 11 showed the result of protein assays.

Table 10. Levels of 8-isoprostaglandins F2α produced in embryos and yolk sacs after culture in different hyperglycemic plus caffeine conditions. Data are expressed in median (range) and * represents $p<0.05$ when means of groups are compared by Least Significant Difference (LSD) test.

	Glucose and Caffeine Level						
	Control (n= 39)	26.70 mM glucose (n=38)	26.70 mM glucose + 1.0 µg/ml caffeine (n= 16)	26.70 mM glucose + 2.5 µg/ml caffeine (n= 16)	26.70 mM glucose + 5.0 µg/ml caffeine (n= 24)	26.70 mM glucose + 10.0 µg/ml caffeine (n= 21)	Kruskal-Wallis Test *p* Value
8-Isoprostaglandins F2α (pg/mm) in embryo	2.68 (0.65-17.46)	5.47 (1.61-38.24)*	1.75 (0.42-4.85)	1.30 (0.24-2.66)*	2.12 (0.63-5.62)	2.20 (0.86-5.62)	<0.01
8-Isoprostaglandins F2α (pg/mm) in yolk sac	1.97 (0.469-9.07)	4.11 (1.07-22.3)*	3.56 (1.11-8.70)	2.51 (0.05-11.06)	0.77 (0.21-1.22)*	0.30 (0.10-0.97)*	<0.001

Table 11. Total protein content in embryos and yolk sacs after 48-h culture in different hyperglycemia plus caffeine levels. Data are expressed in median (range).

	Glucose and Caffeine Level						
	Control	26.70 mM glucose	26.70 mM glucose + 1.0 µg/ml caffeine	26.70 mM glucose + 2.5 µg/ml caffeine	26.70 mM glucose + 5.0 µg/ml caffeine	26.70 mM glucose + 10.0 µg/ml caffeine	Kruskal-Wallis Test *p* Value
Total Protein Content (µg) in Embryo	306.11 (168.32-820.11) (n=29)	305.32 (31.47-800.95) (n=30)	268.84 (158.42-470.11) (n=16)	216.11 (80.79-378.26)* (n=15)	520.75 (272.20-985.79) (n=23)	451.79 (221.50-766.30) (n=22)	<0.01
Total Protein Content (µg) in Yolk Sac	400.40 (195.9-617.20) (n=23)	322.80 (26.20-598.40) (n=24)			333.25 (248.50-461.40) (n=24)	351.55 (179.70-469.00) (n=22)	0.024

Section IV: Discussion and Conclusions

Chapter 8: Discussion

As mentioned in chapter 4, the main objective of this thesis was to answer the question "What are the effects of addition of caffeine on hyperglycemic teratogenesis?" No data on this question has been reported to date. At the same time, it would not be a difficult task to find a habitual caffeine-consuming pregnant lady. Apart from intending to add knowledge on this question, it seems interesting to know whether caffeine would play a role of teratogen to exert additive or synergistic effects to hyperglycemic teratogenesis or it would act as antioxidant to alleviate the malformations induced by hyperglycemia. Between these two possibilities, we hypothesize, based on a series of literature reviews, that caffeine acts as an effective antioxidant at concentrations lower than the threshold teratogenic levels, to scavenge reactive oxygen species generated in hyperglycemic conditions and thus diminish degrees of dysmorphogenesis caused by hyperglycemia.

The project has been divided into three parts: I) to reaffirm the dose-dependent teratogenicity of hyperglycemia. II) to find out the threshold teratogenic caffeine levels III) to assess the combined effects of hyperglycemia and caffeine by using teratogenic glucose doses but sub-teratogenic caffeine dose. Throughout the project, whole embryo culture model (New 1976) was applied to culture conceptus with precise control of concentrations of teratogens in the medium. Embryos after culture were scored morphologically and then sent to measurement of protein content (Lowry et al. 1951), which reflects the extent of growth of embryos. In order to bear out the combined effect is exerted through production and elimination of reactive oxygen species the most reliable

marker of degree of lipid peroxidation, 8-isoprostaglandins F2α, (Morrow et al. 1995), was measured in embryos as well as in yolk sacs.

In this section, discussions will also be divided into parts and carried out according to subtopics as shown below.

Effects of Hyperglycemia on Embryonic Growth and Development

High blood glucose level is a well-known factor causing mal-development of fetuses (Reece et al. 1996). However, the threshold doses leading to anomalies vary from study to study (Reece et al. 1998, Freinkel et al. 1986). Reece et al. observed malformations in two-fold glucose level while Norbert et al did not observe any discernible lesions until in four-fold glucose level. Thus, the first part of this study targeted at: 1) reaffirming the dose-dependent teratogenicity of hyperglycemia; 2) utilizing the whole rat embryo culture model to find out the teratogenic levels of hyperglycemia; 3) laying a fundamental knowledge for the study of the combined effects in the latter part of the project. Apart from carrying out morphological assessment to observe and affected morphological features, the protein assay enabled any effect on the degree of growth in hyperglycemic conditions to be observed.

What are the affected features?

Results reveal the fact that hyperglycemic conditions affect embryonic development in all morphological features that were assessed: yolk sac circulation, allantois, flexion, heart, caudal neural tube, hind brain, mid brain, fore brain, optic system, otic system, number of branchial bars, fore limb, hind limb, total morphology scores, yolk sac diameter, crown rump length, and number of somites. All of these scores obtained probability values less than 0.01 estimated by Kruskal-Wallis test. In sum,

hyperglycemic conditions significantly cause malformations as compared with normal glycemic conditions.

Dose related or not?

Moreover, our data went with the previous findings that hyperglycemia affects embryonic development in a dose dependent fashion (Reece et al. 1998, Soler et al. 1976). Direct negative relationships between morphology scores and degrees of severity of hyperglycemia we observed. These negative trends confirm once again the dose-dependent effect of hyperglycemia on embryonic mal-development.

What is the threshold teratogenic level?

Overall, high blood glucose exerts teratogenic effects above a two fold hyperglycemic level. The total morphology scores obtained in two fold hyperglycemic group show significant difference with $p<0.05$ as compared with the control group. However, this phenomenon does not happen on every morphology feature. Significant differences with $p<0.05$ were only obtained in hind limb, number of somites and total morphology scores when the two-fold hyperglycemic group is compared with the normal glycemic group. While in features of yolk sac circulation, fore brain, optic system, otic system and hind limb features, they do differ as compared with control group in four fold hyperglycemic conditions. For the allantois, flexion, heart, caudal neural tube, hind brain, mid brain, number of branchial bars, fore limb, yolk sac diameter and crown rump length, they differ with control group at eight fold hyperglycemic conditions.

How about growth?

Morphologically speaking, hyperglycemia affects cell differentiation in every feature. However, such direct effects are not consistent for growth as reflected by data from the protein assay. Significant differences cannot

be achieved statistically when the total embryonic protein contents of hyperglycemic groups are compared with the control group. However, when the total protein content of embryos is correlated with the total morphology scores, a correlation coefficient is obtained with a significant value of 0.319, while total protein content of embryos correlated with crown rump length of embryos, with a correlation coefficient of 0.341. In hyperglycemic yolk sacs protein content, results are similar to those of embryonic protein content. No significant protein content difference is shown between any hyperglycemic group and the control group. However, there are again significant correlations between yolk sac protein contents and total morphology scores as well as between yolk sac protein contents and mean yolk sac diameter. The relevant correlation values are 0.155 and 0.397 respectively. Even though significant differences between groups are not achieved, significant correlations do confirm a direct relationship between morphology scores, sizes of yolk sac and embryonic crown rump length with protein content. As predicted from the data, hyperglycemia does affect embryonic growth but in a less sensitive manner.

Mechanistic Considerations in Hyperglycemic Teratology

Until now, it is still not precisely known what mediates the teratogenic effects of hyperglycemia. The following four biochemical pathways have been mentioned to have associations with hyperglycemic teratogenesis: 1) prostanoid synthesis, 2) protein glycation, 3) glucose autoxidation as well as 4) polyol pathway. There are also many factors confirmed to be associated with hyperglycemic embryopathy including yolk sac injury (Pinter et al. 1998, Reece et al. 1986), myo-inositol deficiency (Goldman et al. 1986), genetic factors (Eriksson et al. 1986, 1988), arachidonic acid deficiency (Goldman et al. 1986), accumulation of sorbitol (Weigensberget

et al. 1990; Hod et al. 1986), glycosylated protein formation (Brownlee et al. 1984, Arai et al. 1987), as well as reactive oxygen species production (Chang et al. 1985, Sussman et al. 1988) etc.. Previous studies have shown that a supplement of free oxygen radical scavenging enzymes effectively lowered or even returned the malformation rates of hyperglycemia to normal (Hales et al. 1987, 1988). One study has also shown that hyperglycemia does disturb GSH concentration, which is one of the primary cellular antioxidants (Hales et al. 1991). Concluded from these studies, glucose causes disturbed embryogenesis by generation of oxygen free radicals or lowering of cellular antioxidative ability. Such a postulation is further supported by the evidence that all the four hyperglycemia-associated biochemical pathways result in excessive production of free radicals. In the first part of the present study, we attempt to reaffirm that free oxygen radicals involved in lipid peroxidation are partly responsible for causing embryonic developmental damage in high blood glucose levels conditions by measurement of 8-isoprostaglandins F2α, the most reliable marker for measuring degree of lipid peroxidation mediated by reactive oxygen species (Morrow et al. 1995).

How reliable or how novel is measuring 8-isoprostaglandins F2α in embryos

In the past, there was a lack of methods or, more correctly to say, markers to assess oxidative stress status specifically and sensitively (Halliwell et al. 1987). Recently, the discovery of 8-isoprostaglandins F2α as a product resulting from oxygen free radical attack of lipid, has turned the free radical field to a new page. The reason is that quantitative measurement of isoprostane provides a reliable non-invasive and advanced approach to assess the degree lipid peroxidation and thus oxidative stress status (Morrow et al. 1995). From the best of our knowledge, apart from Wentzel in 1999 (Wentzel et al. 1999) who performed a pilot experiment in

measuring the isorostane level of rat embryos, we are only the second to attempt it. From his study, hyperglycemia of 30 mmol/l increased embryonic lipid peroxidation. Here, lower levels than 30mmol/l were used, which are more clinically applicable. Practically speaking, the isoprostane levels in embryos are two low to be measured. Thus, the recommendation is that stringent adherence to the protocol is essential in extraction of isoprostane.

Do 8-isoprostaglandins F2α levels really correlate with hyperglycemic levels?

For embryonic 8-isoprostaglandins F2α, we have shown embryos cultured in hyperglycemic conditions give a significant increase in levels of 8-isoprostaglandins F2α levels. Multiple comparisons between hyperglycemic groups and control group confirm that embryonic 8-isoprostaglandins F2α starts to differ in four fold hyperglycemic condition when compared with the control. There is also a significant difference in yolk sac 8-isoprostaglandins F2α level between groups, but the level starts to differ in eight fold hyperglycemic conditions as compared with control. As a trial, isoprostane levels in culture medium were also quantified. However, results show that there is no difference in isoprostane levels among hyperglycemic groups. There was a direct negative relationship between embryonic 8-isoprostaglandin F2α activities and morphology, and between yolk sac 8-isoprostaglandins F2α activities and morphology. These relationships have not been reported before and it provides a further support of the role of oxidative stress in hyperglycemia-induced embryonic damages. Our results confirm once again oxidative stress is one of the vital mechanisms causing hyperglycemic embryopathy.

What is recommended? Measuring isoprostane in embryos, yolk sacs or medium?

So far, there are two ways for estimating isoprostane levels in samples.

Without doubt, we do not recommend measuring isoprostane levels in culture media. The reason is that isoprostane levels in media may affect the real isoprostane amount diffused out from embryos during culture. It is not hard to conceive that media composed of both DMEM and rat serum may contain variable amounts of isoprostane secreted by mother rats during anesthesia. Moreover, isoprostane levels vary from rat to rat due to different physiological status. To sum up, culture media is not the best sample to estimate isoprostane levels in this study.

Effects of Caffeine on Embryonic Growth and Development

Acting as one of the most frequently consumed substances (Gilbert 1984), caffeine clearance rate decreases during the course of pregnancy (Aldridge et al. 1981, Knutti et al. 1981, Parsons et al. 1982). Caffeine has been subjected to many studies on its effects on embryonic growth and development since a warning has been put in place by the F. D. A. to suggest pregnant women do not consume caffeine or consume it as little as possible. In animal studies, caffeine has been confirmed as a weak teratogen causing embryonic abnormalities. However, not all investigations have demonstrated the consistent effects. While in human studies, it is found that maternal consumption of caffeine at high doses is associated with numbers of poor pregnancy outcomes (Winick 1998), caffeine consumption could not be related to the frequency of several types of congenital malformations. At the same time, there are data revealing that caffeine is a powerful antioxidant (Kesavan et al. 1985, 1973, 1978, Zhen et al. 1995) with antioxidative ability even more powerful than ascorbic acid (Devasagayam et al. 1996).

As data on the threshold teratogenic dose of caffeine is not consistent and the teratogenic level is animal strain dependent, the second part of the

present study is aimed at finding out the threshold teratogenic level of caffeine for Sprague Dawley rats. Such information could also help to lay a foundation for the latter part of the present study.

What are the affected features?

Based on the non-parametric Kruskal-Wallis test, caffeine exerted teratogenicity in all morphological features that were assessed: yolk sac circulation, allantois, flexion, heart, caudal neural tube, hind brain, mid brain, fore brain, optic system, otic system, branchial bars, fore limb, hind limb, number of somites and hence the total morphology scores of the embryos. Besides, caffeine also reduced embryonic crown rump length and mean yolk sac diameter significantly. Teratogenicity of caffeine was reaffirmed in the present study and its teratogenic effects seem to cover all morphological features that were measured.

What is the threshold teratogenic dose?

Findings from recent research concluded that caffeine is a weak teratogen because it does not express its teratogenic effects below its threshold teratogenic doses, which is approximate 60μg/ml to 80 μg/ml in rodents. Our results also show that significant malformations of morphological features start mostly above a dose of 60μg/ml. However, there were also some exceptional features like yolk sac diameter, number of somites, and hind limb development, where data show that they start to mal-develop at a caffeine dose of 30μg/ml. Hence, these criteria may be more sensitive and fallible to such teratogen. In fact, it is widely known that fetal limb abnormality is one of the most major and easily observed impacts from caffeine. Our results showed that limb development was more sensitive to caffeine. Thus, it is not hard to conceive that the limb is one of the most obvious criteria affected by caffeine.

Dose related or not?

Same as hyperglycemia, direct negative relationships between morphology scores and concentrations of caffeine are reported in Spearman's correlation coefficient with occurrence probability less than 0.01. The negative trends show that the higher the dose of caffeine taken, the worse the development of embryos.

Does caffeine affect growth?

Morphologically speaking, high dose caffeine does affect cell differentiation in every morphological feature. With respect to growth in protein content, data show caffeine exerts effects on growth. Statistical significant difference is achieved among caffeinated groups in yolk sacs. Further, when total protein content of embryos was correlated with total morphology scores, a correlation coefficient was obtained significantly with a value of 0.446. In caffeinated yolk sacs protein content, there was a significant correlation between yolk sac protein contents and total morphology scores. The relevant correlation value was 0.451. As reflected from the data, the higher the dose of caffeine, the less the growth of embryos as well as their yolk sacs.

What caffeine concentration is the hypothesized antioxidant?

The hypothesis of the present project was that caffeine in low concentration would act as an antioxidant to scavenge reactive oxygen radicals effectively and thus correct to a certain extent of damage generated from hyperglycemic conditions. One of the intentions of having the first and the second part of this study was to lay a basis to estimate the amount of glucose as well as the concentration of caffeine to be used in the third part of the study- combined effect of hyperglycemia and caffeine.

As mentioned in discussing hyperglycemic results, four fold glucose

would be taken as the hyperglycemic level for testing the combined effect. The reason of choosing this level is that at this glucose concentration, amount of reactive oxygen radical increases significantly as compared with that in normal glycemic conditions. Besides, this level surely affects morphology and growth of embryos. Hence, it would help to prove, if caffeine would really act as an antioxidant, the amount of reactive oxygen species as well as morphological defects would decrease or even return to normal.

The most critical step was to estimate doses of caffeine to be tested. As confirmed in second part of the study, a high dose of caffeine (equal to or higher than 60 μg/ml caffeine) caused congenital anomalies. At the same time, based on previous findings, researchers have only tested antioxidative power of caffeine at a level of 2μg/ml, where it was sure that this level can exert antioxidative effects (Devasagayam et al. 1996). Besides, the antioxidative ability of caffeine is dose dependent. Thus, thecombined effect was tested by using caffeine levels of 1μg/ml, 2.5μg/ml, 5μg/ml and 10μg/ml. These levels are taken because it had been confirmed that these levels did not induce any teratogenicity whilst it was believed that they could exert their reactive oxygen species-scavenging abilities. More, this level range could be easily achieved habitually, equivalent to taking 1 to 10 cups of coffee per day.

Why there was no quantitation of 8-isoprostaglandins F2α in caffeinated samples?

Embryos and yolk sacs affected by caffeinated conditions were not sent for measurement of levels of 8-isoprostaglandins F2α because of two major reasons. 1) this project was not aimed at finding out if caffeine exerts its teratogenicity through the generation of reactive oxygen species. 2) it is logical to think that if caffeine does really give antioxidative effects, it

would lower the levels of 8-isoprostagladins F2α. However, due to technical problem of the assay, where the lowest detection limit is 3.9pg/ml 8-isopostaglandins F2α, it is not feasible to test the lower-than-baseline level where the baseline level is given by the experimental control. Moreover, it is also certain that if the combined effect corrects or decreases morphological defects and decreases levels of 8-isoprostaglandins F2α, the phenomenon was due to the addition of caffeine and thus caffeine wass acting as an antioxidant at that concentration in this situation.

Combined Effects of Hyperglycemia and Caffeine on Early Embryogenesis

After designing the concentrations for testing the possible combined effects of hyperglycemia and caffeine on early embryogenesis, the study plan was implemented. Results confirmed the hypothesis of this thesis. In this section, assessing criteria will be discussed one by one to analyze effects of sub-teratogenic dose of caffeine on fetal development in hyperglycemic mothers.

Are there any improvements in morphological defects?

Addition of sub-teratogenic amount of caffeine decreased morphological defects caused by hyperglycemic conditions. The above statement is backed up by the data that whilst embryos cultured in 4-fold hyperglycemic conditions gained significantly lower morphological scores as compared to those cultured in normal glycemic conditions, embryos cultured in four-fold glucose media with addition of various subtle amount of caffeine did not differ from those cultured in normal glycemic media. However, they did differ from those cultured in four-fold hyperglycemic medium morphologically in all features assessed. A simple

conclusion could be drawn from here that congenital anomalies caused by high glucose levels could be diminished or corrected by additions of caffeine at millimolar concentrations *in vitro*.

What is the effect on total protein content?

As the protein content of embryos cultured in four fold hyperglycemic condition did not differ from that of control group, due to the insensitivity of the protein level to the teratogen, any effect on total protein content caused by combined effects could not been seen.

Does correction of morphological defects caused by hyperglycemia relate to doses of caffeine?

Increasing doses of caffeine added to the four fold hyperglycemic conditions correlated positively and significantly with morphology scores. Hence, it is observed that caffeine corrected congenital abnormalities caused by hyperglycemia in a dose dependent fashion. If caffeine really corrects such defects through elimination of reactive oxygen species produced by embryos due to environmental hyperglycemia, it agrees with the notion that caffeine scavenges reactive oxygen species in a dose dependent fashion, which would be further justified when 8-isoprostaglandins F2α levels in the combined effect samples were measured.

Many of the recent studies revealed the potentiation of teratogenic effects of caffeine when it was combined with other teratogens (Nash et al. 1989, Kusama et al. 1989, Nakazawa et al. 1985). But, primarily speaking, the present study reveals one of the benefits of caffeine. That is at sub-teratogenic concentrations caffeine can prevent the congenital maldevelopment of embryos in a hyperglycemic environment. As for the underlying correction principle, as mentioned in the hypothesis, it may be

through the scavenging of reactive oxygen species induced by hyperglycemia. Thus, in the coming section, 8-isoprostaglandins F2α levels were examined in hyperglycemic conditions and the combined conditions in order to confirm the viability of this mechanism.

Mechanistic Consideration: Caffeine correct morphological defects through scavenging reactive oxygen species

Caffeine acts as antioxidant?

The logic proposed in the present study is that one of the mechanisms through which hyperglycemia induces its teratogenicity is generation of reactive oxygen species. In fact, as proven in first part of this study, hyperglycemia does cause overproduction of 8-isoprostaglandins F2α. Hence, additions of sub-teratogenic amounts of caffeine, which enable caffeine to act as an antioxidant, could correct morphological defects and scavenge reactive oxygen species caused by hyperglycemia. The 8-isoprostaglandins F2α levels would thus decrease in embryos cultured in media with combined additions of glucose and caffeine.

With respect to embryonic 8-isoprostaglandins F2α levels, again, embryos grown in four-fold hyperglycemic conditions had significant increase in levels of 8-isoprostaglandins F2α as compared with those grew in normal control conditions. In spite of this, embryos cultured in four-fold hyperglycemic conditions with addition of caffeine did not differ in levels of 8-isoprostaglandins F2α as compared with those cultured in normal control conditions but differ in levels of 8-isoprostaglandins F2α as compared with those cultured in four fold hyperglycemic conditions. More, significant negative trends are observed between embryonic isoprostane levels and doses of caffeine added- the more the quantity of caffeine added to same level of hyperglycemic conditions, the lower the

embryonic isoprostane levels. This implies that caffeine exerts its ability of scavenging reactive oxygen species in dose dependent fashion.

As for the yolk sac 8-isoprostaglandins F2α levels, four-fold hyperglycemic yolk sacs show significant increase in isoprostane levels as compared with normal yolk sacs. However, with addition of 1μg/ml caffeine and 2.5 μg/ml caffeine to the same hyperglycemic conditions, yolk sacs isoprostane levels decreased back to normal. With addition of 5μg/ml caffeine and 10μg/ml caffeine to hyperglycemic conditions, yolk sacs isoprostane levels decreased significantly in isoprostane levels as compared with normal yolk sacs. Besides, a direct and significant negative correlation was shown between concentration of isoprostane in yolk sacs and doses of caffeine added to the same hyperglycemic conditions. Predicted from these data, caffeine does scavenge yolk sac reactive oxygen species induced by hyperglycemia in dose dependent manner too. However, the scavenging ability at doses of 5μg/ml and 10μg/ml may be so high that even reactive oxygen species produced in normal conditions were scavenged. The recommended doses of caffeine to be used as antioxidants for scavenging reactive oxygen species produced from four-fold hyperglycemia is 2.5μg/ml or below.

As for isoprostane levels in combined-effect medium, there is no significant difference in any combined effect groups as compared with the control group, even in the four-fold hyperglycemic group as compared with the control group. These findings, as explained before, may be attributed to the fact that culture medium is not a suitable sample type for estimation of 8-isoprostaglandins F2α levels.

From the embryonic and yolk sac isoprostane levels, the conclusion could be drawn that caffeine acts as antioxidant to scavenge reactive oxygen

species induced by hyperglycemia and thus lowers levels of 8-isoprostaglandins F2α.

Data Variation and Outstanding Data in the Present Study

What is the expected protein range in embryos and yolk sacs cultured in the present model?

Based on the data, the expected embryonic protein range in the present study is 100μg to 700μg, while the expected yolk sac protein range is 200μg to 600μg. As described before, the data in the protein assay are not normally distributed. In other words, we discovered that within these ranges, data are distributed randomly with approximately same number of subjects falling in each narrow range.

Are there any data in the protein assay outside the expected range?

Yes, there are some data in the protein assays outside the expected range. There are two embryos cultured in hyperglycemic conditions yielding protein contents approximate to or greater than 1000μg. Also, in the combined effect study, one embryo yielded near 1000μg of total protein. There are also lower extremities, like one in the combined effect study, which had 90.1μg of total protein. To explain these data, we referred to their relevant morphology scores and discovered that upper extremities obtain high total morphology scores like 50 or more. While for low extremities, the relevant total morphology scores are less than 5. Of course, some embryos are estimated to have more than 100μg or less than 700μg even though they really scored low and high, respectively. So, another reason for the phenomenon is that we have to accept that in the protein assay method does exist some variations. As mentioned before, the amount of color varies with different proteins. Some embryos may develop well in some proteins while other develop better in another

proteins. It leads to one variation. Another variation may due to the fact that the color given during protein assay is not strictly proportional to concentration. When combined the above reasons together, it is not difficult to conceive that it is possible to have some errors in this assay.

The same explanation is applied to extremities in yolk sac proteins. There are some data in caffeine groups with yolk sac protein values less than 200μg, and two negative protein value in combined effects group. Again, a few yolk sac protein in caffeine groups larger than 600μg. When referred to their morphology scores, they yielded lower than normal and higher than normal scores.

What are the explanations for the variations in morphological scores?
In the statistical analysis of results, we have already excluded cell masses from the database. So, how come the range of morphological scores is so wide? To explain these variations, we first explain those of control data and then those of experimental groups.

The range of control morphological data is not actually wide. From a scatter plot, we could understand that there are only a very few embryos which scored low. More than 90% of control embryos could yield normal or high scores and the data are mostly skewed to the high scored side. When compared with experimental groups, the number of embryos malformed in control groups is far fewer than those in experimental groups.

In the experimental group, the number of embryos morphologically scored low is large. The simplest reason is that the teratogen added exerted an embryotoxic effect and affects embryonic development. However, we could also observed some did score high and making the

range of data wide. This phenomenon may due to the fact that some embryos may have stronger resistance to the teratogen than others have. At least genetic factor can contribute to their variations in resisting adverse environments.

What are the explanations for the variations in isoprostane data?

As mentioned in the section of Materials and Methods, using the kit provided by Cayman Chemicals yields data higher at high concentrations and lower at low concentrations. This may attribute to the variations of isoprostane data. But the data is still of high availability. We are the second to attempt measurement of isoprostane levels in embryos cultured from this system. The first attempt measured this in the same samples, in the same system and also utilized the same kit as the methodology. The data from these two studies are consistent.

Chapter 9: Conclusions and Future Directions

Conclusions

Based on the logic and the results from the present study, it is concluded that caffeine acts as an effective antioxidant at concentrations lower than its threshold teratogenic levels to scavenge reactive oxygen species induced in hyperglycemic conditions and thus diminish degrees of dysmorphogenesis caused by hyperglycemia.

Future Directions

Even though the situation is not exactly the same in humans as in animals, the present findings would be implicative to clinical cases in a certain extent. In other words, we believe that subtle amount of caffeine may be a good dietary supplement or drug to reduce rates of congenital malformations among infants of hyperglycemic mother. Further reaffirmation on that is expecting and promising.

Appendices

Publications

Chan LY, Chiu PY, Siu NSS and Lau TK. A study of diclofenac-induced teratogenicity during organogenesis using a whole rat embryo culture model. *Human Reproduction*

Chan LY, Chiu PY and Lau TK. A Study of Hypericin-induced teratogenicity during organogenesis using a whole rat embryo culture model. *Fertility and Sterility* 2001 Nov

Chan LYS, Lau TK, Chiu PY, Wong GWK, Leung TN. Levels of cord blood thyroid stimulating hormone after external cephalic version. British Journal of Obstetrics and Gynaecology 2001; 108: 1-5.

Manuscripts in Progress

Lau TK, Chiu PY, Pang MW and Pratten MK. Hyperglycemia increases lipid peroxidation in post-implantation rat embryo explants. *Diabetologia* submitted.

Chiu PY and Lau TK. Sub-teratogenic dose of caffeine alleviates congenital malformations caused by hyperglycemia via lowering increased lipid peroxidation in post-implantation rat embryo explants.

Chan LY, Chiu PY, and Lau TK. Diclofenac-induced developmental damage in rat embryos and increased lipid peroxidation.

References

Acheson KJ, Zahorska-Markiewicz B, Pittet P, Anantharaman K and Jequier E Caffeine and coffee: their influence on metabolic rate and substrate utilization in normal weight and obese individuals. *The American Journal of Clinical Nutrition* 1980; 33(5): 989-97.

Ahmed MU, Thorpe SR and Baynes JW. Identification of Ne-(Carboxymethyl)-lysine as a degradation product of fructose lysine in glycated protein. *The Journal of Biological Chemistry* 1986; 216: 4889-4894.

Ahmed MU, Walla DJ, Thorpe SR and Baynes JW. Oxidative degradation of glucose adducts to protein: Formation of 3-(Ne-lysine)-lactic acid from model compounds and glycated proteins. *The Journal of Biological Chemistry* 1998; 263: 8816-8821.

Aldridge A, Aranda JV and Neims AH. Caffeine metabolism in the newborn. *Clinical Pharmacology and Therapeutics* 1979; 25: 447-453.

Aldridge A, Bailey J and Neims AH . The disposition of caffeine during and after pregnancy. *Seminars in Periatology* 1981; 5(4): 310-4.

Aliverti V, Bonanoni L, Giavine E, Leone G and Mariani L. Extent of fetal ossification as an index of delayed development in teratogenic studies in the rat. *Teratology* 1979; 20: 237-242.

Arai K, Iizuka S, Tada U, Oilkawa and Taniguchi N. Increase in the glycosylated form of erythrocyte Cu-Zn-superoxide dismutase in diabetes and close association of the non-enzymatic glycosylation with the enzyme activity. *Biochimica et Biophysica Acta* 1987; 924: 292-296.

Aranda JV, Collinge JM, Zinman R and Watters G. Maturation of caffeine elimination in infancy. *Archives of Disease in Childhood* 1979; 54: 946-949.

Arnaud MJ. *Caffeine: Perspectives from recent research.* Berlin: Springer Verlag; 1984: 3-38.

Arnaud MJ. *Coffee III: Physiology.* London: Elsevier Applied Publ.; 1988: 33-55.

Arnaud MJ. *Coffee, caffeine and health.* New York: Raven Press; 1983: 43-94.

Arnaud MJ. The pharmacology of caffeine. *Progress in Drug Research* 1987; 31:273-313.

Asahina T, Kashiwagi A, Nishio Y, Ikebuchi M, Harada N, Tanaka Y, Takagi Y, Saeki Y, Kikkawa R and Shigela Y. Impaired activation of glucose oxidation and NADPH supply in human endothelial cells exposed to H2O2 in high-glucose medium. *Diabetes* 1995; 44: 520-526.

Awad JA, Rrberts LJII and Burk RF. Isoprostane- prostaglandin-like compounds formed in vivo independently of cyclooxygenase: use as clinical indicators of oxidant damage. *Gastroenterology Clinics of North America* 1996; 25: 409-427.

Bailey DN, Weibert RT, Naylor AJ and Shaw RF. A study of salicylate and caffeine excretion in the breast milk of two nursing mothers. *Journal of Analytical Toxicology* 1982; 6(2): 64-8.

Bartel H and Gnacikowska M. Histological studies on the influence of caffeine on embryonic development of the limbs in mice. *Folia*

Morphologica 1972; 31: 178.

Battig K and Sullivan FM. The physiological effects of coffee consumption on fetal growth. *American Journal of Obstetrics and Gynaecology* 1987; 157: 1236-1240.

Baynes JW. Role of oxidative stress in development of complications in diabetes. *Diabetes* 1991; 40: 405-412.

Beard RW and Lowy C. The British survey of diabetic pregnancies. *British Journal of Obstetrics and Gynaecology* 1982; 89: 783-786.

Beaulac-Baillargeon L and Desrosiers C. Caffeine-cigarette interaction on fetal growth. *American Journal of Obstetrics and Gynaecology* 1987; 157: 1236-1240.

Berlin CM. Excretion of methylxanthines in human milk. *Seminars in Perinatology* 1981; 5(4): 389-94.

Bernstein RE. Serum and plasma preparation for potassium analysis. Effects of anticoagulants, storage time and temperature before separation, and haemolysis. *South African Journal of Medical Science* 1985; 18: 99-104.

Bertrand M, Girod J and Rigaud MF. Ectrodactyly caused by caffeine in rodents. Role of specific and genetic factors. *Comptes Rendus des Seances Soc Biol Fil* 1970; 164(7): 1488-1489.

Billington WD and Jenkinson EJ. *The early development of mammals*. New York: Cambridge University Press; 1975: 219-232.

Blanchard J and Sawers SJA. The absolute bioavailability of caffeine in

man. *European Journal of Clinical Pharmacology* 1983; 24: 93-98.

Bligh EG and Dyer WJ. A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* 1959; 37: 911-917.

Bonati M, Latini R, Galetti F, Young JF, Tognoni G and Garattini S. Caffeine disposition after oral doses. *Clinical Pharmacology and Therapeutics* 1982; 32: 98-106.

Bonati M, Latini R, Tognoni G, Young JF and Garattini S. Interspecies comparison of in vivo caffeine pharmacokinetics in man, monkey, rabbit, rat, and mouse. *Drug Metabolism Reviews* 1984-85; 15(7): 1355-83.

Borlee L, Lechat MF, Bouckaert A and Misson C. Le café facteur de risque pendant la grossesse? *Louvain Medicine* 1978; 97: 279-284.

Bracco D, Ferrarra JM, Arnaud MJ, Jequier E and Schutz Y. Effects of caffeine on energy metabolism, heart rate, and methylxanthine metabolism in lean and obese women. *American Journal of Physiology* 1995; 269(4 Pt 1): E671-8.

Brazier JL, Ritter J, Berland M, Khenfer D and Faucon G. Pharmacokinetics of caffeine during and after pregnancy. *Developmental Pharmacology and Therapeutics* 1983; 6(5): 315-22.

Brownlee M, Vlassara H and Cerami A. Non-enzymatic glycosylation and the pathogenesis of diabetic complications. *Annals of Internal Medicine* 1984; 101: 527-537.

Bruce A and Bray D. *Molecular Biology of the Cell.* New Yolk: Garland Publishing Inc.; 1983: 745-748.

Brudenell M and Doddridge MC. *Diabetic Pregnancy.* Edinburgh London Melbourne and New York: Longman Group UK Limited; 1989: 1-17.

Bunker ML and McWillams M. Caffeine content of common beverages. *Journal of American Diabetic Association* 1979; 75(1): 28-32.

Burg AW. Physiological disposition of caffeine. *Drug Metabolism Reviews* 1975; 4(2): 199-228.

Carrier O, Pons G, Rey E, Richard MO Moran C, Badoual J and Olive G. Maturation of caffeine metabolic pathways in infancy. *Clinical Pharmacology and Therapeutics* 1988; 44: 145-151.

Chang JCF, Ulrich PC, Bucala R and Cerami A. Detection of an advanced glycosylation product bound to protein in situ. *The Journal of Biological Chemistry* 1985; 260: 7970-7974.

Cheraskin E and Ringsdorf WM. Blood-glucose levels after caffeine. *Lancet* 1968; 2(7569): 689.

Chung CS, Myrianthopoulos NC. Factors affecting risks of congenital malformations. *Birth Defects* 1975; 11: 23.

Cockroft DL. Culture media for postimplantation embryos. *Reproductive Toxicology* 1991; 5: 223-228.

Collins TFX, Welsh JJ, Black TN and Collins E. A comprehensive study of

the teratogenic potential of caffeine in rats when given by oral intubation. *Regulatory Toxicology and Pharmacology* 1981; 1: 355-378.

Collins TFX, Welsh JJ, Black TN and Collins EV. A study of the teratogenic potential of caffeine given by oral intubation of rats. *Regulatory Toxicology and Pharmacology* 1981; 1: 355.

Collins TFX, Welsh JJ, Black TN and Ruggles DI. A study of the teratogenic potential of caffeine ingested in drinking water. *Food and Chemical Toxicology* 1983; 21: 763-777.

Collins TFX, Welsh JJ, Black TN and Ruggles DI. *Alternative dietary practices and nutritional abuses in pregnancy. Proceedings of a Workshop.* New York: National Academy Press; 1981: 97-107.

Collins TFX. Review of reproduction and teratology studies of caffeine. FDA by lines 1979; 9: 352-373.

Comline RS and Silver M. Placental transfer of blood gases. *British Medical Bulletin* 1975; 37: 237-249.

Daly JW. *Caffeine, coffee and health.* New York: Raven Press; 1993: 97.

DeHaan RL. *A symposium on the chemical basis of development.* Baltimore: Johns Hopkins Press; 1958: 339-373.

DeHaan RL. Modification of cell-migration patterns in the early chick embryo. *Proceedings of the National Academy of Sciences* 1958; 44: 32-37.

Delpech A and Delpech B. Expression of hyaluronic acid-binding glycoprotein, hyaluronectin, in the developing rat embryo. *Developmental*

Biology 1984; 101: 391-400.

Deuchar EM. Regeneration of amputated limb-buds in early rat embryos. *Journal of Embryology and Experimental Morphology* 1976; 35: 345-354.

Devasagaam TPA and Kesavan PC. Radioprotective and antioxidant action of caffeine: Mechanistic considerations. *Indian Journal of Experimental Biology* 1996; 34: 291-297.

Devasagayam TPA and Kesavan PC. Radioprotective and antioxidant action of caffeine: Mechanistic considerations. *Indian Journal of Experimental Biology* 1996; 34: 291-297.

Devasagayam TPA, Kamat JP, Mohan H and Kesavan PC. Caffeine as an antioxidant: inhibition of lipid peroxidation induced by reactive oxygen species. *Biochimica et Biophysica Acta* 1996; 1282: 63-70.

Dodd SL, Brooks E, Powers SK and Tulley R The effects of caffeine on graded exercise performance in caffeine naïve versus habituated subjects. European *Journal of Applied Physiology and Occupational Physiology* 1991; 62(6): 424-9.

Drury MI, Green AT and Strong JM. Pregnancy complicated by clinical diabetes mellitus: A study of 600 pregnancies. *American Journal of Obstetrics and Gynaecology* 1977; 49: 519.

Drury MI, Greene AT and Stronge JM. Pregnancy complicated by clinical diabetes mellitus: a study of 600 pregnancies. *Obstetrics and Gynaecology* 1977; 49: 519-522.

Druzin M, Socol M, Murata Y and Manning FA. Fetal bradycardia and death following profound experimental hypoglycemia in the primate fetus. *Proceedings/ SGI* 1980.

Dyer DG, Blackledge JA, Thorpe SR and Baynes JW. Formation of pentosidine during non-enzymatic browning of proteins by glucose: Identification of glucose and other carbohydrates as possible precursors of pentosidine in vivo. *The Journal of Biological Chemistry* 1991; 166: 11654-11660.

Elmazar MMA , McElhatton PR and Sullivan FM. Studies on the teratogenic effects of different oral preparations of caffeine in mice. *Human Toxicology* 1981; 1: 53-63.

Elmazar MMA, McElhatton PR and Sullivan FM. Studies on the teratogenic effects of different oral preparations of caffeine in mice. *Toxicology* 1983; 28: 427.

Elmazar MMA, McElhatton PR and Sullivan FM. Studies on the teratogenic effects of different oral preparations of caffeine in mice. *Toxicology* 1982; 23: 57-71.

Emanuel C. Blood glucose levels after caffeine. *Lancet* 1968; 2(7569): 689.

Eriksson RSM, Thunberg L, Eriksson UJ. Effects of interrupted insulin treatment on fetal outcome of pregnant diabetic rats. *Diabetes* 1989; 38: 764-772.

Eriksson UJ and Borg LAH. Diabetes and embryonic malformations: Role of substrate-induced free oxygen radical production for

dysmorphogenesis in cultured rat embryos. *Diabetes* 1993; 42: 411-419.

Eriksson UJ and Borg LAH. Protection by free oxygen radical scavenging enzymes against glucose-induced embryonic malformations in vitro. *Diabetologia* 1991; 34: 325-331.

Eriksson UJ and Borg LAH. Protection by oxygen radical scavenging enzymes against glucose-induced embryonic malformations in vitro. *Diabetologia* 1991; 34:325-331.

Eriksson UJ and Siman CM. Pregnant diabetic rats fed the antioxidant butylated hydroxytoluene show decreased occurrence of malformation in the offspring. *Diabetes* 1996; 45:1497-1502.

Eriksson UJ, Bone AJ, Turnbull DM and Baird JD. Timed interruption of insulin therapy in diabetic BB/E rat pregnancy: effects on maternal metabolism and fetal outcome. *Acta Endocrinology* 1989; 28: 292-293.

Eriksson UJ, Borg HLA and Forsberg H. Can fetal loss be prevented? The biochemical basis of diabetic embryopathy. *Diabetes Reviews* 1996; 4(1): 49-69.

Eriksson UJ, Dahlstrom VE and Lithell HO. Diabetes in pregnancy: Influence of genetic background and maternal diabetic state on the incidence of skeletal malformations in the fetal rat. *Acta Endocrinologica* 1986; 277: 66-73.

Eriksson UJ. Importance of genetic predisposition and maternal environment for the occurrence of congenital malformations in offspring of diabetic rats. *Teratology* 1988; 37: 365-374.

Fabry GVM, Delhaise F and Picard JJ. Morphogenesis and quantification of the development of post-implantation mouse embryos. *Toxicology in Vitro* 1990; 4(2): 149-156.

Fabry GVM, Delhaise F and Picard JJ. Morphogenesis and quantification of the development of post-implantation mouse embryos. *Toxicology in Vitro* 1990; 4(2): 149-156.

Fabry GVM, Delhaise F and Picard JJ. Morphogenesis and quantification of the development of post-implantation mouse embryos. *Toxicology in Vitro* 1990; 4(2): 149-156.

Fadel RAR and Persaud TVN. Craniofacial ossification in the rat following prenatal exposure to alcohol, acetaldehyde and caffeine. *Research Communications in Substances Abuse* 1991; 12: 181-184.

Fadel RAR and Persaud TVN. Effects of alcohol and caffeine on cultured whole rat embryos. *Acta Anatomica* 1992; 144: 114-119.

Farooqi Z and Kesavan PC. Radioprotection by caffeine pre- and post-treatment in the bone marrow chromosomes of mice given whole-body gamma-irradiation. *Mutation Research* 1992; 269(2): 225-230.

Food and Drug Research Laboratoy Inc. Teratologic Evaluation of FDA 71-44(Caffeine) National Technical Information Service Report No. PB-221-803, 1973, January.

Food and Drug Research Labs., Inc. Teratologic Evaluation of FDA 71-44(Caffeine) National Technical Information Service Report No.

PB-221-827, 1973, January.

Forsberg H, Eirksson UJ and Melefors O. Beta-hydroxybutyrate increases reactive oxygen species in late but not in early postimplantation embryonic cells in vitro. *Diabetes* 1998; 47:255-262.

Fredholm BB. Adenosine, adenosine receptors, and the actions of caffeine. *Pharmacology and Toxicology* 1995; 76: 93-101.

Fredholm BB. *The methylxanthine beverages and foods: Chemistry, consumption and health effects*. New York: Spiller; 1984: 303.

Fredholm BB. *The methylxanthine beverages and foods: Chemistry, consumption and health effects*. New York: Spiller; 1984: 331.

Fredholm BB. *The methylxanthine beverages and foods: Chemistry, consumption and health effects*. New York: Spiller; 1984: 365.

Freinkel N, Cockroft DL, Lewis NJ, Gorman L, Skazawa S, Phillips LS and Shambaugh III GE. The 1986 McCollum award lecture: Fuel mediated teratogenesis of glucose, ketones, or somatomedin inhibitor during rat embryo culture. *The American of Clinical Nutrition* 1986; 44: 986-995.

Fujii T and Nakatsuda T. Potentiating effects of caffeine on teratogenicity of alkylating agents in mice. *Teratology* 1983; 28: 29-33.

Fujii T and Nishimura H. Adverse effects of prolonged administration of caffeine on rat fetus. *Toxicology and Applied Pharmacology* 1972; 22: 449-457.

Fujii T and Nishimura H. Teratogenic actions of some methylated xanthines in mice. *Okajimas Fol Anat Jap* 1969; 19: 134-138.

Fujii T, Nishimura H. Reduction infrequency of fetopathic effects of caffeine in mice by pretreatment with propranolol. *Teratology* 1974; 10: 149-152.

Fujii T, Sasaki N and Nishimura H. Teratogenicity of caffeine in mice related to its mode of administration. *Japanese Journal of Pharmacology* 1969; 19: 134.

Furuhashi N, Sato S, Suzuki M, Hiruta M, Tanaka M and Takahashi T. Effects of caffeine ingestion during pregnancy. *Gynecologic and Obstetric Investigation* 1985; 19: 187-191.

Gilani SH and Persaud TVN. Chick embryonic development following exposure to caffeine and nicotine. *Anatomischer Anzeiger* 1986; 161: 23-26.

Gilani SH and Persaud TVN. Embryopathic effects of ethanol and caffeine in the chick. *Anatomischer Anzeiger* 1985; 158: 231-235.

Gilbert EF and Pistey WR. Effect on the offspring of repeated caffeine administration to pregnant rats. *Journal of Reproduction and Fertility* 1973; 34: 495-499.

Gilbert RM. *The methylxanthine beverages and foods: Chemistry, consumption, and health effects*. New Yolk. 1984:185-214.

Global health situation V) Noncommunicable diseases, diability. *Weekly Epidemiological Record* 1993; 68: 93-99.

Goldman A, Dicker D , Feldberg D, Yeshaya A, Smauel N and Karp M.

Pregnancy outcome in patients with insulin-dependent diabetes mellitus with preconceptional diabetic control: A comparative study. *American Journal of Obstetrics and Gynaecology* 1986; 155: 293.

Goldstein A and Warren R. Passage of Caffeine into human gonadal and fetal tissue. *Biochemical Pharmacology* 1962; 11: 166-168.

Grandhee SK and Monnier VM. Mechanism of formation of the Maillard protein crosslink pentosidine: Glucose, fructose and ascorbate as pentosidine precursors. *The Journal of Biological Chemistry* 1991; 266: 11649-11653.

Greene DA, Lattimer SA and Sima AAF. Sorbitol, phosphoinositides, and sodium-potassium-ATPase in the pathogenesis of diabetic complications. *The New England Journal of Medicine* 1987; 316: 599-606.

Group d'Ethde des Risques Teratogenes. Experimental Teratogenesis: study of caffeine with mice. *Therapie* 1969; 24: 575.

Hagay Z, Weiss U and Zusman I. Prevention of diabetic embryopathy by overexpression of the free radical scavenging enzyme, superoxide dismutase, in transgenic mouse embryos. *American Journal of Obstetrics and Gynecology* 1995; 122: 254.

Hagay ZJ, Weiss Y, Zusman I, Peled-Kamar M, Reece EA, Eriksson UJ, Groner Y. Prevention of diabetes-associated embryopathy by overexpression of the free radical scavenger copper zinc superoxide dismutase in transgenic mouse embryos. *American Journal of Obstetrics and Gynaecology* 1995; 173: 1036-1041)

Hales BF and Brown H. The effect of in vivo glutathione depletion with buthionine sulfoximine on rat embryo development. *Teratology* 1991; 44: 251-257.

Halliwell B and Grootveld M. The measurement of free radical reactions in humans. Some thoughts for future experimentation. *FEBS Letters* 1987; 213: 9-14.

Halliwell B. Drug antioxidant effects: A basis for drug selection? *Drug* 1991; 42(4): 569-605.

Halliwell B. Establishing the significance and optimal intake of dietary antioxidants: The biomarker concept. *Nutrition Reviews* 1999; 57(4): 104-113.

Halliwell B. Free radicals, reactive oxygen species and human disease: a critical evaluation with special reference to atherosclerosis. *British Journal of Experimental Pathology* 1989; 70: 737-757.

Halliwell B. How to charaterise a biological antioxidant. *Free Radical Research Communications* 1990; 9: 1-32.

Hanson U, Persson B and Thunsell S. Relationship between haemoglobin A_{1c} in early type I (insulin-dependent) diabetic pregnancy and the occurrence of spontaneous abortion and fetal malformation in Sweden. *Diabetologia* 1990; 100: 33.

Harris C, Namkung MJ and Juchau MR. Regulation of intracellular glutathione in rat embryos and visceral yolk sacs and its effects on 2-nitrosofluorene-induced malformations in the whole embryo culture

system. *Toxicology and Applied Pharmacology* 1987; 88: 141-152.

Harris C, Stark KL and Juchau MR. Glutathione status and the incidence of neural tube defects elicited by direct acting teratogens in vitro. *Teratology* 1988; 37: 577-590.

Harris M, Eastman R and Cowie C. Symptoms of sensory neuropathy in adults with NIDDM in the U.S. population. *Diabetes Care* 1993; 16: 1446.

Hasegawa M, Yamada S and Hirayama C. Fasting plasma caffeine level in cirrhotic patients: relation to plasma levels of catecholamines and renin activity. *Hepatology* 1989; 10(6): 973-977.

Heinonen OP, Slone D and Shapiro S. *Birth defects and drugs in pregnancy.* Littleton: Publishing Sciences; 1977.

Henderson GI, Baskin GS, Frosto TA and Schenker S. Interactive effects of ethanol and caffeine on rat fetal hepatocyte replication and EGF receptor expression. *Alcohol Clinical and Experimental Research* 1991; 15: 175-180.

Hirsh K. *The methylxanthine beverages and foods: Chemistry, consumption and health effects.* New York: Spiller; 1984: 235.

Hod M, Star S and Passoneau J. Effect of hyperglycemia on sorbitol and myo-inositol content of cultured rat conceptus: Failure of aldose reductase inhibitors to modify myo-inositol depletion and dysmorphogenesis. *Biochemical and Biophysical Research* 1986; 140: 974-980.

Hohman TC and Beg MA. Diabetic complications: progress in the development of treatments. *Exp Opin Invest Drugs* 1994; 3: 1041-1049.

Hughes JR, Higgins ST, Bickel WK, Hunt WK, Fenwick JW, Gulliver SB and Mireault GC. Caffeine self-administration, withdrawal, and adverse effects among coffee drinkers. *Archives of General Psychiatry* 1991; 48: 611-617.

Hunt JV, Dean RT and Wolff SP. Hydroxyl radical production and autoxidative glycosylation: Glucose autoxidation as the cause of protein damage in the experimental glycation model of diabetes mellitus and ageing. *The Biochemical Journal* 1988; 256: 205-212.

Hunt JV, Dean RT and Wolff SP. Hydroxyl radical production and autoxidative glycosylation: glucose autoxidation as the cause of protein damage in the experimental glycation model of diabetes mellitus and ageing. *The Biochemical Journal* 1988; 256: 205-212.

Ikeda GJ, Sapienza PP, McGinnis ML, Bragg LE, Walsh JJ and Collins TFX. Blood levels of caffeine and results of fetal examination after oral administration of caffeine to pregnant rats. *Journal of Applied Toxicology* 1982; 2(6): 307-314.

Imamura SI, Kimura M, Hiratsuka E, Takao A and Matsuoka R. Effect of caffeine on expression of cardiac myosin heavy chain gene in adult hypothyroid and fetal rats. *Circulation Research* 1992; 71: 1031-1038.

International Coffee Organization. Federal Republic of Germany. Coffee consumption habits trend data 1980 to 1990. ICO, ED PC 97/91 (E), July, 1991.

Iwase T, Arishima K, Ohyama N, Inazawa K, Iwase Y, Ikeda Y, Shirai M,

Yamamoto M, Somiya H and Eguch Y. In vitro study of teratogenic effects of caffeine on cultured rat embryos and embryonic cells. *Journal of Veterinary Medical Science* 1994; 56(3): 619-921.

Jacobson JD and Cousins L. A population-based study of maternal and perinatal outcome in patients with gestational diabetes. *American Journal of Obstetrics and Gynaecology* 1989; 161: 981-986.

Jacobson MF, Goldman AS and Syme RH. Coffee and birth defects. *Lancet* 1981; i: 1415-1416.

James JE. *Caffeine and health*. New York: Academic Press; 1991: 219-244.

Jensen M, Koszalka TR and Brent RL. Production of congenital malformations using tissue antisera. XV. Reichert's membrane and visceral yolk sac antisera. *Developmental Biology* 1975; 42: 1-12.

Jensh RP, Koszalka TR, Jensen M, Biddle L and Brent RL. Morphological alterations in the parietal yolk-sac of the rat from the 12th to the 19th day of gestation. *Journal of Embryology and Experimental Morphology* 1977; 39: 9-21.

Jiang ZY, Woollard ACS and Wolff SP. Hydrogen peroxide production during experimental protein glycation. *FEBS Letters* 1990; 268: 69-71.

Joeres RP, Vermeulen NP and Breimer DD. Influence of the rate of hepatic portal vein infusion on hexobarbital pharmacokinetics in the rat. *Pharmacology* 1998; 36(3): 210-216.

Jovanovic L and Peterson CM. Management of the pregnant, insulin-dependent of diabetic pregnant women. *Diabetic Care* 1980; 3:63-68.

Kesavan PC and Natarajan AT. Protection and potentiation of radiaion clastogenesis by caffeine: nature of possible initial events. *Mutation Research* 1985; 143 (1 Pt 2): 61-68.

Kesavan PC and Powers EL. Differnetial modification of oxic and anoxic components of radiation damage in Bacillus megaterium spores by caffeine. *Interational Journal of Radiation Biology* 1985; 48(2): 223-233.

Kesavan PC, Trasi S and Ahmad A. Modification of barley seed radiosensitivity by posttreatment with caffeine. I. Effect of post-irradiation heat shock and nature of hydration. *International Radiation in Biology* 1973; 24(6): 581-587.

King H and Rewers M. Diabetes in adults is now a third world problem. *Bull World Health Organizatoion* 1991; 89: 643-648.

Kirkinen P, Jouppila O, Koivula A, Vuori J and Pukka M. The effect of caffeine on placental and fetal blood flow in human pregnancy. *American Journal of Obstetrics and Gynecology* 1983; 147: 929-942.

Kitzmiller JL, Buchanan TA, Kjos S, Combs CA and Ratner RE. Preconception care of diabetes, congenital malformation, and spontaneous abortions. *Diabetes Care* 1996; 19: 514.

Kitzmiller JL, Gavin LA and Gin GD. Preconception care of diabetes: Glycemic control prevents congenital anomalies. *The Journal of the American Medical Association* 1991; 263: 731-736.

Klein NW, Minghetti PP, Jackson SK and Vogler MA. Serum protein

depletion by cultured rat embryos. *Journal of Experimental Zoology* 1978; 203(2): 313-318.

Knoche C and Konig J. Zur pranatalen Toxizitat von diphenyl-pyralin-8-chlortheophyllinat unter berucksichtingung con erfahrungen mit thalidomid und coffein. *Arzneim Forsch* 1964; 14: 415-424.

Knutti R, Rothweiler H and Schlatter C. Effect of pregnancy on the pharmacokinetics of caffeine. *European Journal of Clinical Pharmacology* 1981; 21(2): 121-6.

Kochhar DM. The use of in vitro procedures in teratology. *Teratology* 1975; 2: 274-287.

Kurppa K, Holmberg PC, Kuosma E and Saxen L. Coffee consumption during pregnancy and selected congenital malformations: A nationwide case-control study. *American Journal of Public Health* 1983; 73: 1387-1399.

Kusama T, Suguira N, Kai M and Yoshizawa Y. Combined effects of radiation and caffeine on embryonic development in mice. *Radiation Research* 1989; 117: 273-281.

Lawrence E. *Henderson's English-Chinese Dictionary of Biological Terms*. 1st ed. UK: Longman; 1989: 234.

Lawrence E. *Henderson's English-Chinese Dictionary of Biological Terms*. 1st ed. UK: Longman; 1989: 520.

Lechat MF, Borlee I, Bouckaert A and Misson C. Caffeine study. *Science* 1980; 207: 1296.

Linn S, Schoenbaum S, Monson RR, Rosner B, Stubblefield G and Ryan KJ.

No association between coffee consumption and adverse outcomes of pregnancy. *The New England Journal of Medicine* 1982; 306: 141-145.

Lowry OH, Rosebrough NJ and Farr AL. Protein measurement with the folin phenol reagents. *The Journal of Biological Chemistry* 1951; 193: 165-270.

Lowry OH, Rosebrough NJ, Farr AL. Protein measurement with the folin phenol reagents. *The Journal of Biological Chemistry* 1951; 193: 165-270.

Lowy C. Management of diabetes in pregnancy. *Diabetes Metabolism Reviews* 1993; 147: 9.

Malins J. *Carbohydrate metabolism in pregnancy and the newborn*. New York: Springer Verleg, Berlin, Heidelberg; 1979: 229-246.

Marret S, Delpech B, Girard N, Leroy A, Maingonnat C, Menard JF and Fessard C. Caffeine decreases glial cell number and increases hyaluronan secretion in newborn rat brain cultures. *Pediatric Research* 1993; 34: 716-719.

Marret S, Gresens P, Van-Maele-Fabry G, Picard J and Evrard P. Caffeine-induced disturbances of early neurogenesis in whole mouse embryo cultures. *Brain Research* 1997; 773: 213-216.

Marti J. An overview: Maternal nicotine and caffeine consumption and offspring outcome. *Neurobehavioral Toxicology and Teratology* 1982; 4: 421-424.

McDonald AD, Armstrong BG and Sloan M. Cigarette, alcohol, and coffee consumption and congenital defects. *American Journal of Public Health* 1992; 82: 91-93.

Mills J, Baker L, Goldman AS. Malformations in infants of diabetic mothers occur before the seventh gestational week. *Diabetes* 1989; 28: 292-293.

Milon H, Guidoux R and Antonioli JA. *Caffee III: Physiology* London: Elsevier Applied Science; 1988: 81.

Mimouni F, Miodovnik M, Siddiqi TA, Berk MA, Wittekind C and Tsang RC. High spontaneous premature labor rated in insulin-dependent diabetic pregnant women: an association with poor glycemic control and urogenital infection. *Obstetrics and Gynaecology* 1988; 1972: 175-180.

Miodovnik M, Skillman C, Holroyde JC, Butler JB, Wendel JS and Siddiqi TA. Elevated maternal gycohemoglobin in early pregnancy and spontaneous abortion among insulin-independent diabetic women. *American Journal of Obstetrics and Gynaecology* 1985; 153: 439-442.

Molsted-Pedersen L and Pedersen JF. Congenital malformations in diabetic pregnancies. Clinical viewpoints. *Acta Pediatica Scandinavia* 1985; 320 supplement: 79-84.

Morris MB and Weinstein L. Caffeine and the fetus: is trouble brewing? *American Journal of Obstetrics and Gynaecology* 1981; 140: 607-608.

Morrow JD and Roberts LJ. Mass Spectrometry of prostanoids: F2-isoprostane produced by non-cyclooxygenase free radical-catalyzed mechanism. *Methods in Enzymology* 1994; 233: 163-174.

Morrow JD, Harris TM and Roberts II LJ. Noncyclooxygenase oxidative

formation of a series of novel prostaglandins: analytical ramifications for measurement of eicosanoids. *Analytical Biochemistry* 1990; 184: 1-10.

Morrow JD, Hill KE and Burk RF. A series of prostaglandin F2-like compounds are produced in vivo in humans by a non-cyclooxygenase, free radical-catalyzed mechainism. *National Academy of Sciences* 1990; 87: 9383-9387.

Mulhivill JJ. Caffeine as teratogen and mutagen. *Teratology* 1973; 8: 69-73.

Muller WU, Streffer C and Fischer-Lahdo C. Effects of a combination of X-raysand caffeine on perimplantation mouse embryos in vitro. *Radiation and Environmental Biophysics* 1983; 22: 85-93.

Muller WU, Streffer C and Wurm R. Supraadditive formation of micronuclei in preimplantation mouse embryos in vitro after combined treatment with X-rays and caffeine. *Teratogenesis, Carcinogenesis, and Mutagenesis* 1985; 5: 123-131.

Muller WU. Toxicity of various combinations of X-rays, caffeine, and mercury in mouse embryos. *International Journal of Radiation Biology* 1989; 56: 315-323.

Mulvihill JJ. Caffeine as teratogen and mutagen. *Teratology* 1973; 8: 69-72.

Murphy SJ and Benjamin CP. The effects of coffee on mouse development. *Microbiology Letters* 1981; 17: 91.

Nash JE and Persaud TVN. Influence of nicotine and caffeine on rat embryonic development. *Histology and Histopathology* 1988; 3: 377-388.

Nash JE and Persaud TVN. Influence of nicotine and caffeine on skeletal development in the rat. *Anatomischer Anzeiger* 1989; 168: 109-126.

Nash JE and Persaud TVN. Reproductive and teratological risks of caffeine. *Anatomischer Anzeiger* 1988; 167: 265-270.

Natkatsuda T, Hanada S and Fujii T. Potentiating effects of methylxanthines on teratogenicity of mitomycin C in mice. *Teratology* 1983; 18: 243-247.

Nehlig A, Daval JL and Debry G. Caffeine and central nervous system: Mechanisms of action, biochemical, metabolic and psychostimulant effects. *Brain Research Reviews* 1992; 17: 139-170.

Nelson MM and Forfar JD. Associations between drugs administered during pregnancy and congenital abnormalities of the fetus. *British Medical Journal* 1971; i: 523-527.

New DAT and Cockroft DL. A rotating bottle culture method with continuous replacement of the gas phase. *Experientia* 1978; 35(1): 138-140.
New DAT, Coppola PT and Cockroft DL. Improved development of headfold rat embryos in culture resulting from low oxygen and modifications of the culture serum. *Journal of Reproduction and Fertility* 1976; 48: 219-222.

New DAT. *In vitro Methods in Toxicology*. London: Academic Press; 1992: 431-445.

New DAT. Whole-embryo culture and the study of mammalian embryos during organogenesis. *Biological Reviews* 1978; 53: 81-122.

Nishimura H and Nakai K. Congenital malformations in offspring of mice treated with caffeine. *Society for Experimental Biology and Medicine* 1960; 104: 140.

Nogee L, deMello D, Dehner L and Colten H. Brief report deficiency of pulmonary surfactant protein B in congenital alveolar proteinosis. *New England Journal of Medicine* 1993; 328: 406.

Nolen GA. A reproduction/ teratology study of brewed and instant decaffeinated coffee. *Journal of Toxicology and Environmental Health* 1982; 10: 769-782.

Nolen GA. *Issues and Reviews in Teratology*. New York: Plenum Press; 1988: 305-350.

Nolen GA. The effect of brewed and instant coffee on reproduction and teratogenesis in the rat. *Toxicology and Applied Pharmacology* 1981; 58: 171-183.

Nygard O, Resum H, Ueland PM, Stenvold I, Nordrehaug JE, Kvale G and Vollset SE. Coffee consumption and plasma total homocysteine: The Hordaland homocysteine study. *American Journal of Clinical Nutrition* 1997; 136: 65.

Oates JN, Abell DA, Beischer NA and Broomhall GR. Maternal glucose tolerance during pregnancy with excessive size infants. *Obstetrics and Gynaecology* 1980; 55: 184-186.

Oda A, Bannai C, Yamaoka T, Katori T, Matsushima T, Yamashita K. Inactivation of Cu, Zn-superoxide dismutase by in vitro glycosylation and in erythrocytes of diabetic subjects. *Hormone and Metabolic Research* 1994; 26: 1-4.

Odor DL and Blandau RJ. Light and electron microscopic observation on the cervical epithelium of the rabbit. *American Journal of Anatomy* 1988; 181(3): 289-319.

Odor DL, Gaddum RP, Rumery RE and Blandau RJ. Cyclic variations in the oviuductal ciliated cells during the menstrual cycle and after estrogen treatment in the pig-tailed monkey, Macaca nemestrina. *Anatomical Record* 1980; 198(1): 35-57.

Office of Population Censuses and Surveys. OPCS Monitor MB3 81/4 London Government Statistical Service, 1981.

Olsen J, Overvad K and Frische G. Coffee consumption, birth-weight, and reproductive failures. *Epidemiology* 1991; 2: 370-374.

Omachi R. The pathogenesis and prevention of diabetic nephropathy. *Western Journal of Medicine* 1986; 145: 222.

Palm PE, Arnold EP, Rachwall PC, Leyczeck JC, Teague KW and Kensler CJ. Evaluation of the teratogenic potential of fresh brewed coffee and caffeine in the rat. *Toxicology and Applied Pharmacology* 1978; 64: 1813-1817.

Pariente-Khayat A, Pons G, Rey E, Richard MO, D'Athis P, Moran C, Badoual J and Olive G. Caffeine acetylator phenotyping during

maturation in infants. *Pediatric Research* 1991; 29:492-495.

Parsons WDand Pelletier JG. Delayed elimination of caffeine by women in the last 2 weeks of pregnancy. *Canadian Medical Association Journal* 1982; 127(5): 377-80.

Pinter E, Reece EA and Leranth CZ. Yolk sac failure in embryopathy due to hyperlgycemia: Ultrastructural analytical of yolk sac differentiation associated with embryopathy in rat conceptus under hyperglycemic conditions. *Teratology* 1986; 33: 73.

Pinter E, Reece EA and Pgbum P. Relative essential fatty acid deficiency in hyperglycemia-induced embryopathy. *American Journal of Obstetrics and Gynecology* 1988; 159: 1484-1490.

Pinter E, Reece EA, Leranth CZ, Segura MG, Hobbins JC, Mahoney MJ and Naftolin F. Arachidonic acid prevents hyperglycemia-associated yolk sac damage and embryopathy. *American Journal of Obstetrics and Gynecology* 1986; 155(4): 691-702.

Pradelles P, Grassi J and Maclouf J. Enzyme immunoassays of eicosanoids using acetylcholinesterase. *Methods in Enzymology* 1990; 187: 24-34.

Proudfoot J, Barden A, Mori TA, Burke V, Croft KD, Beilin LJ and Puddey IB. Measurement of urinary F2-isoprostane as markers of in vivo lipid peroxidation- a comparison of enzyme immunoassay with gas chromatography/ mass spectrometry. *Analytical Biochemistry* 1999; 272: 209-215.

Pryor WA, Stanley JP and Blair E. Autoxidation of polyunsaturated fatty acids: A suggested mechanism for the formation of TBA-reactive materials

from prostaglandin like endoperoxides. *Lipids* 1976; 11: 370-379.

Purves D and Sullivan FM. *Caffeine, coffee and health.* New York: Raven Press, Ltd.; 1993: 317-342.

Raghu B and Kesavan PC. Radiobiology and Bacillus megaterium spores: Physiochemical events involving oxygen and caffeine. *Indian Journal of Experimental Biology* 1986; 24(12): 742-746.

Reece EA and Eriksson UJ. The pathogenesis of diabetes-associated congenital malformations. *Obstetrics and Gynecology Clinics of North America* 1996; 23(1): 29-45.

Reece EA, Homko CJ, Wu YK and Wiznitzer A. The role of free radicals on membrane lipids in diabetes-induced congenital malformations. *Journal of the Society for Gynecologic Investigation* 1998; 5(4): 178-187.

Reece EA, Khandelwal M, Wu YK and Borenstein M. Dietary intake of myo-inositol and neural tube defects in offspring of diabetic rats. *American Journal of Obstetrics and Gynaecology* 1997; 176(3): 536-539.

Reece EA, Pinter E and Leranth CA. Yolk sac failure in embryopathy due to hyperlgycemia: An ultrastructural analysis. *Teratology* 1986; 32: 363.

Roberts LJII and Morrow JD. Isoprostanes: Novel markers of endogenous lipid peroxidation and potential mediators of oxidant injury. *Annals of the Academy of Science, NY* 1994; 744: 237-242.

Rosenberg L, Mitchell AA, Shapiro S and Slone D. Selected birth defects in relation to caffeine-containing beverages. *The Journal of the American*

Medical Association 1982; 247: 1429-1432.

Rosenn B, Miodovnik M, Combs CA, Khoury J and Siddiqi TA. Poor glycemic control and antepartum obstetric complications in women with insulin-dependent diabetes. *International Journal of Obstetrics and Gynaecology* 1993; 43: 21-28.

Ross CP and Persaud TVN. Cardiovascular primordium in the rat embryo following in utero exposure to alcohol and caffeine. *Canadian Journal of Cardiology* 1986; 2(3): 160-163.

Ross CP and Persaud TVN. Craniofacial and limb development in early rat embyos following in utero exposure to ethanol and caffeine. *Anatomischer Anzeiger* 1990; 170: 9-14.

Ross CP and Persaud TVN. Early embryonic development in the rat following in utero exposure to alcohol and caffeine. *Histology and Histopathology* 1986; 1: 13-17.

Ross CP and Persaud TVN. Neural tube defects in early embyo following maternal treatment with ethanol and caffeine. *Anatomischer Anzeiger* 1989; 169: 247-252.

Rusnak SL and Driscoll SG. Congenital spinal anomalies in infants of diabetic mothers. *Pediatrics* 1965; 35: 989.

Schlegel R and Tardee AB. Caffeine induced uncoupling of mitosis from the completion of DNA replication in mammalian cells. *Science* 1986; 232: 1264-1266.

Schreiner CM, Zimmerman EF, Wee EL and Scott WJ. Caffeine effects on cyclic AMP levels I the mouse embryonic limb and palate in vitro. *Teratology* 1986; 34: 21-27.

Scott WJ. Caffeine-induced limb malformations: Description of malformations and quantitation of placenta transfer. *Teratology* 1983; 28: 427.

Sell DR and Monnier VM. End stage renal disease and diabetes catalyze the formation of a pentose-derived crosslink from ageing human collagen. *The Journal of Clinical Investigation* 1990; 85: 380-384.

Sell DR and Monnier VM. Structure elucidation of a senescence cross-link from human extracellular matrix: Implication of pentoses in the ageing process. *The Journal of Biological Chemistry* 1989; 264: 21597-21602.

Shepard TH. *Catalog of teratogenic agents*. 3rd ed. Baltimore: The John Hopkins University Press; 1980.

Shi X and Dalal NS. Antioxidant behaviour of caffeine: efficient scavenging of hydroxyl radicals. *Food and Chemical Toxicology* 1991; 29(1): 1-6.

Siman CM and Eriksson UJ. Vitamin E decreases the occurrence of malformations in the offspring of diabetic rats. *Diabetes* 1997; 46:1054-1061.

Singer DB. *Diabetes mellitus in pregnancy*. New York: Churchill Livingstone Inc.; 1995: 115.

Sivan E, Reece EA and Wu YK. Dietary vitamin E prophylaxis and diabetic embryopathy: morphologic and biochemical analysis. *American Journal of*

Obstetrics and Gynaecology 1996; 175:793-799.

Sivan E, Wu YK and Homko CJ. Dietary vitamin E propylaxis and diabetic embryopathy: Morphological, biochemical and molecular analyses. *American Journal of Obstetrics and Gynecology* 1996; 175(4 Pt 1): 793-799.

Smith SE, McElhatton PR and Sullivan FM. Effects of administering caffeine to pregnant rats either as a single daily dose or as divided doses four times a day. *Food and Chemical Toxicology* 1987; 25: 125.

Smith WL. Prostaglandin biosynthesis and its compartmentation in vascular smooth muscle and endothelial cells. *Annual Review of Physiology* 1986; 48: 251-262.

Snigorska B and Bartel J. Studies in the teratogenic influence of caffeine in the fetuses of white mice. *Folia Morphologica* 1970; 29: 353.

Soler NG, Walsh CH and Malins JM. Congenital malformations in infants of diabetic mothers. *The Quarterly Journal of Medicine* 1976; 45: 303.

Spindel E. *The methylxanthine beverages and foods: Chemistry, consumption and health effects*. New York: Spiller; 1984: 355.

Steele CE and New DAT. Serum variants causing the formation of double hearts and other abnormalities in explanted rat embryos. *Journal of Embryology and Experimental Morphology* 1974; 31: 707-719.

Steele CE. Improved development of rat 'egg-cylinders' in vitro as a result of fusion of the heart primordial. *Nature New Biology* 1972; 237: 150-151.

Sussman EJ, William GT and Soper KA. Diagnosis of diabetic eye disease. *The Journal of the American Medical Association* 1982; 247: 3231.

Sussman I and Matschinsky FM. Diabetes affects sorbitol and myo-inositol levels of neuroectodermal tissue during embryogenesis in rat. *Diabetes* 1988; 37: 974-981.

Tanaka H, Iwasaki S, Arima M and Nakazawa K. Effects of combinations of maternal agents on the fetal cerebrum in rat: Ethanol or caffeine with X-irradiation in utero. *Brain Development* 1985; 7: 10-20.

Taylor PM, Wofson JH, Bright NH. Hyperbilirubinemia in infants of diabetic mothers. *Biology and the Neonate* 1963; 5: 289.

Thomson AM, Billewicz WZ and Hytten FE. The assessment of fetal growth. *Journal of Obstetrics and Gynaecology British Commonwealth* 1968; 75: 903-916.

Tikkanen J and Heinonen OP. Cardiovascular malformations and organic solvent exposure during pregnancy in Finland. *American Journal of International Medicine* 1988; 14: 1-8.

Trocino RA, Akazawa S and Ishibashi M. Significance of glutathione depletion and oxidative stress in early embryogenesis in glucose-induced rat embryo culture. *Diabetes* 1995; 44:992-998.

Tsai TD and Barish ME. Imaging of caffeine-inducible release of intracellular calcium in cultured embryonic mouse telencephalic neurons. *Journal of Neurobiology* 1995; 27: 252-265.

Van't Hoff W, Caffeine in pregnancy. *Lancet* 1982; 1(8279): 1020.

Vonkeman H, Nugteren DH, van Dorp DA. The action of prostaglandin 15-hydroxydehydrogenase on various prostaglandins. *Biochimica et Biophysica Acta* 1969; 187(4): 581-583.

Wang Z, Ciabattoni G, Creminon C, Lawson J, Fitzgerald GA, Patrono C and Maclouf J. Immunological characterization of urinary 8-epi-prostaglandins F2 alpha excretion in man. *Journal of Pharmacology and Experimental Therapeutics* 1995; 275: 94-100.

Warso MA and Lands WEM. Lipid peroxidation in relation to prostacyclin and thromboxane physiology and pathophysiology. *British Medical Bulletin* 1983; 39: 277-280.

Weigensberg MJ, Palmer GFJ and Freinkel N. Uptake of myo-inositol by early-somite rat conceptus: transport kinetics and effects of hyperglycemia. *Diabetes* 1990; 39: 575-582.

Wentzel P and Eriksson UJ. Antioxidants diminish developmental damage induced by high glucose and cyclooxygenase inhibitors in rat embryos in vitro. *Diabetes* 1998; 47:677-684.

Wentzel P, Welsh N and Eriksson UJ. Developmental damage, increased lipid peroxidation, diminished cycloocygenase-2 gene expression and lowered prostaglandin E2 levels in rat embryos exposed to a diabetic environment. *Diabetes* 1999; 48: 813-819.

White P. *Joslin's diabetes mellitus*. Philadelphia: Lea and Febiger; 1971: 583.

WHO Expert Committee on Diabetes 1980 Second report. WHO Technical

Report Series 646, Geneva.

William OH. *Diabetes mellitus in pregnancy*. New York: Churchill Livingstone Inc.; 1995: 372.

Williamson JR, Chang K, Frangos M, Hasan KS, Ido Y, Kawamura T, Nyengaard JR, Van Den Enden M, Kilo C and Tilton RG. Hyperglycemic pseudohypoxia and diabetic complications. *Diabetes* 1993; 42: 801-813.

Wilson JG and Scott WJ. *Caffeine. Perspectives from recent research.* Berlin: Springer Verlag; 1984: 165-187.

Winick M. *Caffeine.* Boca Raton, Fla: CRC Press; 1998; 357-362.

Wolff SP and Dean RT. Glucose autoxidation and protein modification: The potential role of "autoxidative glycosylation' in diabetes. *The Biochemical Journal* 1987; 245: 243-250.

Yang X, Borg LA and Eriksson UJ. Altered metabolism and superoxide generation in neural tissue of rat embryos exposed to high glucose. *American Journal of Physiology* 1997; 272:E173-E180.

Yochim JM. Development of the progestional uterus: Metabolic aspects. *Biology of Reproduction* 1975; 12: 106-133.

Zhen W and Vaughan AT. Effect of caffeine on radiation-induced apoptosis in TK6 cells. *Radiation Research* 1995; 141(2): 170-175.